Environmental fate and transport of a new energetic material CL-20

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U.S. Army Edgewood Chemical Biological Center

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ACRONYMS/ABBREVIATIONS

AC	acetone control
ACN	acetonitrile
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
APG	Aberdeen Proving Ground
ARS	Agricultural Research Service
a.s.e.	asymptotic standard error
ASTM	American Society of Testing and Materials
ATCLP	Adapted Toxicity Characteristic Leaching Procedure
BAF	bioaccumulations factor
BDL	below detection limit
°C	degrees Centigrade
CAS	Chemical Abstract Service
CEC	Cation Exchange Capacity
CESMU	Controlled Environment Soil-core Microcosm Unit
CI	Confidence Interval
COPC	contaminant of potential concern
d	day
DAD	diode array detector
DHA	dehydrogenase activity
EC	effective concentration
Eco-SSL	Ecological Soil Screening Level
EM	energetic material
EPA	Environmental Protection Agency
ERA	Ecological Risk Assessment
FRT	Folsomia Reproduction Test
G	Gravity
g	gram
h	hour
HDPE	high-density polyethylene
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high melting explosive)
HPLC	High Performance Liquid Chromatography
IC	inhibiting concentration
ID	inner diameter
ISO	International Standardization Organization
kg	kilogram
λ	wavelength
LC	Lethal Concentration
LOD	Limit of Detection
LOAEC	Lowest Observed Adverse Effect Concentration
LOEC	Lowest Observed Effect Concentration
LSD	Least Significant Difference
M	Moles (or molar)
m	meter
µ	micro
mg	milligram
min	minute

mL	milliliter
mm	millimeter
η	nano
NC	negative control
ND	not determined
NOAEC	No Observed Adverse Effect Concentration
NOEC	No Observed Effect Concentration
NRC	National Research Council of Canada
OECD	Organization for Economic Cooperation and Development
OM	organic matter
p	probability
PNA	potential nitrification activity
PTFE	polytetrafluoroethylene
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive)
RH	Relative Humidity
RO	Reverse Osmosis
RSD	Relative Standard Deviation
SE	Standard Error
sec	second
SECOFASE	**S**ublethal **E**ffects of **C**hemicals **o**n **F**auna in the **S**oil **E**cosystem
S/N	Signal to Noise ratio
SSL	Sassafras Sandy Loam
TCLP	Toxicity Characteristic Leaching Procedure
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene
USDA	U.S. Department of Agriculture
USEPA	U.S. Environmental Protection Agency
UV	Ultraviolet
WHC	Water Holding Capacity
x	times
2-ADNT	2-amino-4,6-dinitrotoluene
4-ADNT	4-amino-2,6-dinitrotoluene
2,4-DANT	2,4-diamino-6-nitrotoluene
2,6-DANT	2,6-diamino-4-nitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene

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2. Executive Summary

This project was undertaken to assess the potential ecological impacts of the release of CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) into the environment. These investigations produced experimental data on the toxicity of cyclic nitramine CL-20 to terrestrial plants, soil invertebrates, and aquatic species. Terrestrial ecotoxicological investigations were specifically designed to meet the USEPA criteria for ecological soil screening level (Eco-SSL) derivation. A natural soil, Sassafras sandy loam, was used in all toxicity studies. Sassafras sandy loam (SSL) had low organic matter and clay contents, fulfilling the USEPA Eco-SSL requirement for plant and soil invertebrate testing of using soil with characteristics that support relatively high contaminant bioavailability. We also examined the effects on ecotoxicity of weathering and aging of CL-20 in soil, investigated the effect of CL-20 on the indigenous soil microinvertebrate community, examined the effect of CL-20 on litter decomposition, plus investigated the transport and fate of CL-20 in the environment using a system of standardized intact soil-core microcosms to assess potential toxicity in groundwater, fate endpoints, including persistence and mobility, and plant bioaccumulation potential. Special consideration was given to the effects of weathering and aging of CL-20 in soil on the exposure of ecological receptors in order to more closely simulate the potential exposure effects in the field. All ecotoxicological parameters were established using analytically determined CL-20 concentrations.

Toxicity of CL-20 to terrestrial plants was assessed according to protocols of American Society for Testing and Materials (ASTM) standard guide for conducting terrestrial plant toxicity tests, and the USEPA early seedling growth test. Results of our phytotoxicity studies showed that CL-20 did not adversely affect terrestrial plant growth or seedling emergence of *Medicago sativa* L. (alfalfa; dicot), *Echinochloa crusgalli* L. (Japanese millet; monocot), and *Lolium perenne* L. (perennial ryegrass; monocot) up to and including 7,856 mg kg^{-1}. These results were obtained in a soil that supports relatively high bioavailability of CL-20, with species that represent a wide variety of plant genera and habitats. Alfalfa and perennial ryegrass are crop plants, whereas Japanese millet grows naturally and can tolerate relatively wet habitats. These factors in combination with our test results suggest that CL-20 is relatively non-toxic to the majority of terrestrial plants.

Results from soil invertebrate testing showed that reproduction measurement endpoints were more sensitive indicators of ecotoxicity than was adult survival. The

reproduction endpoints for earthworms, potworms, and collembola were production of juveniles, plus the additional reproduction endpoint of cocoon production for earthworms. The results of standardized single-species toxicity tests showed that toxicity of CL-20 to the earthworm *E. fetida* (ISO 11268-2), enchytraeid worm *E. crypticus* (ISO 16387), and collembolan *F. candida* (ISO 11267) in natural SSL soil were orders of magnitude greater than that of the explosives RDX, HMX, or TNT. Results of toxicity tests with CL-20 weathered and aged in SSL soils showed significantly increased toxicity for potworm *E. crypticus*, but decreased toxicity for collembolan *F. candida*. These changes in toxicity for soil invertebrates exposed in treatments containing CL-20 weathered and aged in soil strongly indicate that the soil chemical environment was altered during the 20-week weathering and aging period, similar to changes that can occur in soil vadose zone environments in the field. Our findings of increased toxicity to *E. crypticus* of CL-20 weathered and aged in soil clearly show that additional studies are required to investigate the toxicity of the CL-20 degradation products. Further investigations of the degradation of CL-20 in soil and the resulting soil toxicity should have a weathering and aging component, so that the level of persistence and long-term impact on the ecotoxicity of these degradation products may also be assessed. Resolving CL-20 degradation pathways that lead to formation of toxic products, their fate in aerobic soils, and assessment of the individual toxicities of degradation products to soil receptors require further investigations to better understand the mechanisms of CL-20 toxicity in the soil vadose zone.

The results of the microcosm study showed that indigenous microarthropod and nematode communities exhibited different sensitivities to CL-20 in soil. Total numbers of nematodes were either unaffected, or increased, through the highest CL-20 treatments tested. In contrast, CL-20 significantly adversely affected the microarthropod community after 4 and 8 weeks of exposure, but the total number of microarthropods was not significantly different from controls at the end of the 12-week exposure. Analysis of community structure revealed greater sensitivities to CL-20 of predatory mesostigmatid mites and predatory nematodes. The observed decreases in respective predatory groups of the microinvertebrate community can potentially result in disruption of the soil food web structure. Effects of weathering and aging of CL-20 in soil on the exposure of soil receptors were investigated in this study by chronosequential harvesting of subset replicate-units to determine potential alterations in bioavailability and resulting toxicity of CL-20 to microinvertebrates. Toxicity of CL-20 decreased overtime for Collembola, prostigmatid mites, and predatory nematodes, but briefly increased for oribatid mites after 8 weeks before returning to approximately initial-level after 12 weeks. These results showed the complexity of possible interactions among the physicochemical fate processes during weathering and aging of CL-20 in soil, changes in bioavailability of parent material and its possible degradation products, and the soil receptor-specific sensitivities of diverse groups of the soil invertebrate community. Results of the concurrent litter decomposition investigation indicated indirectly that soil biotic activity controlling the rate of litter decomposition was either unaffected or stimulated by exposure to CL-20 in SSL soil up to and including 10,300 mg kg^{-1} initial (8238 mg kg^{-1} final after 8 months). The two litter grazing groups, oribatid mites and collembola, jointly comprised approximately 40% of the microarthropod community throughout the study. Furthermore, dominance of bacterivore and fungivore nematodes among the nematode community and the increases in their absolute numbers that were sustained throughout the study suggest indirectly that availability of their respective food sources, bacteria and fungi, were also unaffected, or increased in soil CL-20 treatments.

We assessed the risks to aquatic ecosystems from potential direct release into aquatic habitats, contaminated surface soil runoff or erosion of contaminated soil into water bodies, and transport of CL-20 to groundwater, by quantifying the toxicity of CL-20 to ecologically relevant aquatic organisms using standardized single-species toxicity tests. The toxicity of CL-20 to aquatic species was investigated using algae (*S. capricornutum*), Ceriodaphnia (*C. dubia*), and fathead minnows (*P. promelas*), in independent tests. Chemical analyses were done to determine soluble CL-20 in media. Results of aquatic toxicity assays indicated that relevant ecological receptors may be negatively impacted by exposure to CL-20 if the compound was released into the environment. This risk to aquatic species is significant both from potential direct release into aquatic habitats, and from contaminated surface soil runoff or erosion of contaminated soil into water bodies. Several factors indicate that the release of CL-20 to aquatic ecosystems can potentially cause significant ecological damage in affected sites. These factors include high toxicity of CL-20 to heterotrophic aquatic species, and its potentially stimulating effect on autotrophic algae which can lead to eutrophycation of aquatic habitats. Ecological impacts of CL-20 on aquatic receptors can be further exacerbated by some of the CL-20 fate characteristics including its apparent persistence in soil, and the long-term mobility of aqueous CL-20. Overall results of this investigation strongly indicate that accidental release of CL-20 into the environment can have detrimental effects on resident aquatic ecological receptors.

Assessment of transport and fate of CL-20 in the soil vadose zone were determined using an improved system, the Controlled Environment Soil-core Microcosm Unit (CESMU), adapted from the USEPA and ASTM standard soil microcosm designs. An important modification to the original design was the application of tension at the bottom of each soil column to mimic field conditions and prevent the build-up of water within columns, which can otherwise change the chemical, physical, and biological properties of soil. Intact soil-cores of the SSL soil type used in toxicity tests were used in the transport and fate study. CL-20 concentrates of 98.4 or 9,555 mg kg^{-1} were prepared in SSL soil, and amended atop the surface of the intact SSL soil-cores. During the 35-week study, CL-20 concentrations in soil were periodically determined for soil collected from different depths, using acetonitrile extraction method. Ryegrass plants were grown in each of the soil-cores, harvested at the time of soil sampling, weighed, and placed in a –80ºC freezer until they were analyzed. Soil-cores were sectioned incrementally by depth, and the CL-20 concentration in each soil section was analytically determined. The fate of that portion of CL-20 that partitions into water was assessed by analyzing both the leachates throughout the study period, and the plant tissues of perennial ryegrass. These investigations showed that potential transport and fate of CL-20 in the soil vadose zone was effectively assessed using the CESMU method. Precipitation (simulated rainfall) caused CL-20 to migrate downward in the soil, with concentrations of CL-20 rapidly decreasing with increasing depth of the soil core. Exceptionally small quantities of CL-20 migrated below the 12-cm soil depth of the intact soil core, indicating that CL-20 is not expected to migrate to substantial depth in solid crystal form. Transport of CL-20 in the soil vadose zone was primarily due to solubilization and subsequent partitioning between soil and pore water. Concentrations of CL-20 in soil leachates never reached the CL-20 limit of solubility but increased over 11 weeks, becoming relatively stable at approximately 0.5 and 2 mg L^{-1} in 98.4 and 9,555 mg kg^{-1} soil treatments, respectively. These results suggest a reasonably high potential for long-term transport of CL-20 to groundwater. Soil physico-chemical parameters

that enhance the carrying or sorptive capacity of soil for CL-20 may retard but not eliminate CL-20 migration. However, soil factors that enhance the rate of dissolution of CL-20 in soil, or its aqueous solubility in soil pore water, will increase its potential for transport into groundwater. The BAF values of 0.09 and 0.02 established in studies with ryegrass grown in 98.4 or 9,555 mg kg^{-1} CL-20 treatments for 35 weeks, respectively suggest low bioaccumulations potential for CL-20 in plants.

Draft Eco-SSL values were developed using ecotoxicological benchmarks for soil invertebrates based on analytically determined soil concentration of CL-20 in single-species definitive toxicity tests. Draft Eco-SSLs for soil invertebrates were derived for CL-20 freshly amended into SSL soil, and for CL-20 weathered and aged in SSL soil. CL-20 was not phytotoxic up to nominal 10,000 mg kg^{-1}, the highest concentration tested in the Limit Test with the three plant species. Consequently, no draft Eco-SSL for terrestrial plants was developed for CL-20. The toxicity benchmark values and reports detailing these studies will be provided to the USEPA Ecological Soil Screening Level Workgroup for review. Results will undergo quality control review by the Eco-SSL Task Group before inclusion in the Eco-SSL database, and before acceptance for derivation of Ecological Soil Screening Level (Eco-SSL) for CL-20.

It is recommended that the information documented in this report be considered by those who manufacture CL-20, as well as potential users, risk assessors, site managers, and especially CL-20 Project Managers, prior to transition of CL-20 to military products that currently use energetic materials RDX, HMX, or TNT.

3. Objectives

The general goal of the research was to investigate fate, transport through the vadose zone, and environmental effects of CL-20 on terrestrial plants, soil organisms, and aquatic species, to assess the potential impact of the release of this compound into the environment. Environmental effects of CL-20 were determined using both standardized single-species toxicity assays for soil invertebrates, plants, and aquatic species, and an experimental approach designed to assess community-level effects. The effects of weathering and aging of chemical:soil mixtures was incorporated into the experimental design to better assess the bioavailability potential of CL-20 undergoing biotic and abiotic degradation and transformation processes. Toxicity endpoints (e.g., survival, growth, and reproduction) have been correlated with the concentrations of acetonitrile-extractable CL-20, with the ultimate goal of developing ecotoxicological parameters for CL-20 based on concentration-response relationships that describe its bioavailability for ecologically relevant soil and aquatic biota. Transport and fate was assessed using a system of standardized intact soil-core microcosms. Data collected in this study was used to determine transport of CL-20 through the soil vadose zone, potential toxicity in groundwater, fate endpoints such as persistence and mobility, and bioaccumulation factors.

The goals of this research were achieved by addressing the following technical objectives:

1) Establishing toxicological benchmarks for soil invertebrates and terrestrial plants using standardized single-species toxicity tests with soil that supports relatively high bioavailability of CL-20.
2) Examining the effect of CL-20 on the indigenous soil microinvertebrate community in soil that supports relatively high bioavailability of CL-20, using a microcosm assay designed to provide ecotoxicological data for validating the ecotoxicity data from standardized single-species toxicity tests, and bridge the gap between single-species toxicity tests and field studies.
3) Examining the effect of CL-20 on litter decomposition (one of the most integrating processes within the soil ecosystem) in order to assess the potential risks to carbon and nutrient cycling in the soil ecosystem that may result from accidental release of CL-20 into a soil environment.
4) Examining the effects of simulated weathering and aging of CL-20 in soil on ecotoxicity.
5) Assessing the risks to aquatic ecosystems from potential direct release into aquatic habitats, contaminated surface soil runoff or erosion of contaminated soil into water bodies, and transport of CL-20 to groundwater, by quantifying the toxicity of CL-20 to ecologically relevant aquatic organisms using standardized single-species toxicity tests.
6) Determining transport and fate of CL-20 using a modified standardized intact soil-core microcosm system designed to assess the risks of CL-20 release in the soil vadose zone, including its potential for downward migration in solid crystal or solubilized forms, the potential for transport to groundwater, and the potential for bioaccumulation in terrestrial plants.
7) Determining draft Ecological Soil Screening Level values for ecologically relevant soil receptors for use in Ecological Risk Assessment in the event of accidental release of CL-20 in the environment.

4. Project Background

Polyazapolycyclic caged polynitramine 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (HNIW or CL-20) is a new energetic material whose ecological effects on soil biota, transport through the soil vadose zone, potential ecotoxicity in groundwater, and fate endpoints such as persistence, mobility, and bioaccumulation factors are unknown. Because CL-20 is being considered as a potential replacement for existing high explosive and propellant materials, the potential impacts of its release in the environment needed investigation.

Meaningful management of sites where potential release of CL-20 may occur requires understanding of the ecotoxicology of this chemical. Development of ecological benchmarks for CL-20 such as those required for developing an Ecological Soil Screening Level (Eco-SSL) are the key steps in establishing guidance for monitoring concentrations of CL-20 released into the environment. An Eco-SSL is defined as soil concentration of this chemical which, if not exceeded, will theoretically be protective of unacceptable harmful effects from that chemical in wide-ranging terrestrial ecosystems (USEPA, 2005). Draft Eco-SSL values for CL-20 were derived in this project from data generated in laboratory toxicity tests with different test organisms, representing the vast array of naturally occurring ecological receptors. In order that Eco-SSL is appropriately effects-based, receptor responses were coupled with appropriate

measures of chemical exposure that integrated chemical bioavailability. Chemical exposure was then correlated with biological responses. The ecotoxicity of CL-20 was investigated in this project using an integrated ecotoxicology and chemistry approach. This approach required that toxicity tests were carried out in parallel with chemical analyses, allowing us to link the environmental behavior and fate of CL-20 with its ecotoxicity.

5. Material and Methods

The general technical approach for the study is presented in Figure 1. The research was conducted in two phases. In phase one, we quantified the toxicity of CL-20 to soil invertebrates, terrestrial plants, and aquatic species. Both range-finding and definitive toxicity tests utilized internationally accepted standardized methods. This included amending natural soil, Sassafras sandy loam (SSL), with a range of CL-20 concentrations. A portion of amended soil was subjected to a simulated weathering and aging process prior to definitive bioassays. Aquatic bioassays were conducted with both soil elutriates, and directly-amended test media. The range of CL-20 concentrations tested provides a continuum of exposure concentrations from "no effect" to "bioaccumulation levels" to "lethal levels," ensuring investigation of biological and chemical relationships across orders of magnitude in exposure concentrations.

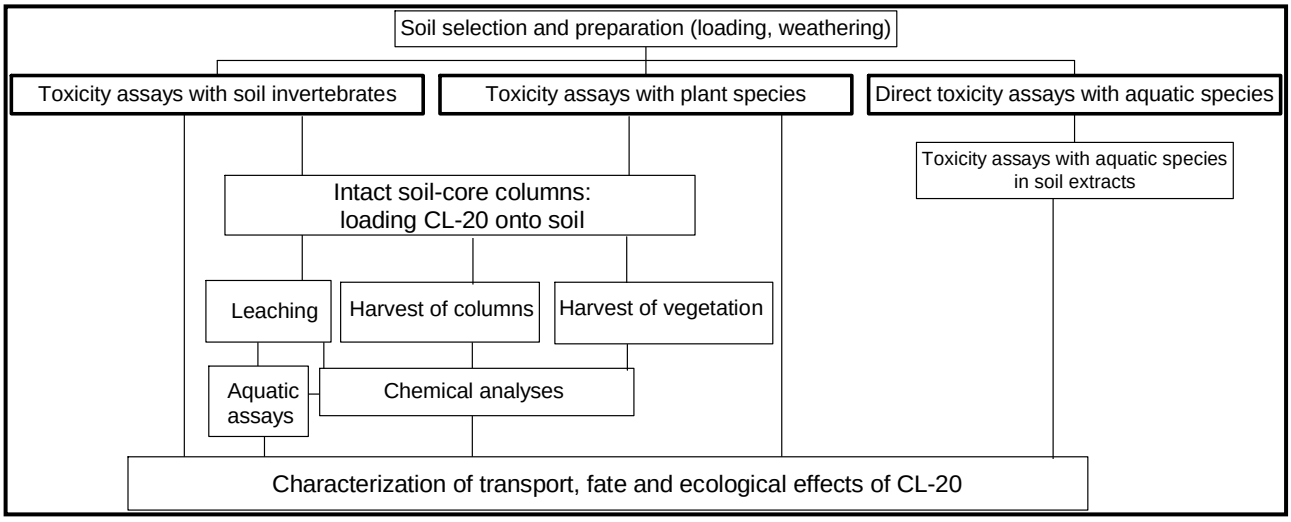

Figure 1. Overview of the technical approach.

In phase two, we characterized transport and fate of CL-20 using standardized intact soil core microcosms. Concentration levels were selected based on the results of the definitive toxicity tests conducted in phase one. Transport and fate were assessed in the soil by depth and in leachates throughout the study period, and in plant tissue at the end of the 35-week study. Aquatic toxicity assays were conducted with leachates to supplement information on potential toxicity of CL-20 in groundwater following transport through the vadose zone of soil.

5.1 Test Soil

For ecological risk assessment purposes, and in particular for developing ecotoxicological values protective of soil biota, we selected a natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (USDA/ARS 1999; SSL) collected from a grassy field at Aberdeen Proving Ground, MD. The physical and chemical characteristics of this soil (low clay and organic matter contents) potentially support a relatively high level of chemical bioavailability (USEPA 2005). For soil collection, vegetation and the litter layer were removed and the top six inches of the A horizon were then excavated. The soil was sieved through a 5-mm mesh screen, air-dried for at least 72 h and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Soil prepared in this manner was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg^{-1}	5.5
Organic matter %	1.2
pH	5.2

5.2 Test Materials

Crystalline CL-20 (CAS 135285-90-4; ε-isomer, purity 99.3%) was obtained from ATK Thiokol Propulsion (Ogden, UT, USA). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$, CAS 7787-56-6, purity 99.99%, Alfa Aesar, Ward Hill, MA, USA) was used as the positive control in the soil invertebrate tests. Boric acid (H_3BO_3, CAS 10043-35-3, purity 99.99%, Alfa Aesar, Ward, MA, USA) was used as the positive control in the plant tests. Acetone (CH_3COCH_3, CAS 67-64-1, HPLC Grade, Fisher Scientific, Pittsburgh, PA, USA) was used for preparing CL-20 solutions for soil amendments. Acetonitrile (CH_3CN, CAS 75-05-8, HPLC Grade, Pharmco, Brookfield, CT, USA) was used for chemical extractions and for subsequent analyses. Methanol (CH_3OH, CAS 67-56-1, Chromatography grade, purity 99.9%, Pharmco, Brookfield, CT, USA) was used in determinations by HPLC. Ethanol (CH_3CH_2OH, CAS 64-17-5, purity 99.98%; Pharmco, Brookfield, CT, USA) was used as preservative for potworms and soil microarthropods. Calcium chloride ($CaCl_2 \cdot 2H_2O$, CAS 10043-52-4, reagent grade 100%, J.T. Baker, Phillipsburg, NJ, USA) was used as flocculant and sodium bisulfate monohydrate ($NaHSO_4 \cdot H_2O$, CAS 10034-88-5, purity 99%, SIGMA-ALDRICH, St. Louis, MO, USA) was used to acidify stock solutions in preparation of chemical extracts from soil for determinations by HPLC. Rose Bengal

biological stain ($C_{20}H_2Cl_4I_4Na_2O_5$, CAS 632-68-8, dye content 80%, Fisher Scientific, Pittsburgh, PA, USA) was used for staining potworm tissues. Unless otherwise specified, American Society of Testing and Materials (ASTM) Type I water (ASTM, 2004a) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA, USA) was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM Type II water (ASTM 2004a), analytical reagent grade nitric acid 1% (volume/volume), then with ASTM Type I water.

Algae media (USEPA 1994) for aquatic toxicity tests was prepared from five nutrient stock solutions using reagent grade chemicals. One-mL of each stock solution was added to approximately 900 mL of distilled ASTM Type I water and mixed, then each made to 1-L of stock solution, respectively:

Stock 1. Magnesium chloride (12.16g L^{-1}), $MgCl_2 \cdot 6H_2O$, CAS 7791-18-6, 99.8%, Fisher Scientific, Pittsburgh, PA, USA,
Calcium chloride (4.4 g L^{-1}), $CaCl_2 \cdot 2H_2O$, CAS 10035-04-8, 99%, SIGMA-ALDRICH, St. Louis, MO, USA
Sodium nitrate (25.5 g L^{-1}), $NaNO_3$, CAS 7631-99-4,100.2%, J.T. Baker, Phillipsburg, NJ, USA

Stock 2. Magnesium sulfate (14.7 g L^{-1}), $MgSO_4$, CAS 10034-99-8, 100.9%, SIGMA-ALDRICH, St. Louis, MO, USA

Stock 3. Potassium phosphate (1.04 g L^{-1}), K_2HPO_4, CAS 7758-11-4, 99.4%, Fisher Scientific, Pittsburgh, PA, USA

Stock 4. Sodium bicarbonate (15g L^{-1}), $NaHCO_3$, CAS 144-55-8, 100%, SIGMA-ALDRICH, St. Louis, MO, USA

Stock 5. Boric acid (185.6 mg L^{-1}), H_3BO_3, CAS 10043-35-3, 100.8%, J.T. Baker, Phillipsburg, NJ, USA
Manganese chloride (416 mg L^{-1}), $MnCl_2 \cdot 4H_2O$, CAS 13446-34-9, >98%, SIGMA-ALDRICH, St. Louis, MO, USA
Zinc chloride (3.28 mg L^{-1}), $ZnCl_2$, CAS 7646-85-7, 98.5%, J.T. Baker, Phillipsburg, NJ, USA
Iron chloride, (159.8 mg L^{-1}), $FeCl_3 \cdot 6H_2O$, CAS 10025-77-1,>99%, Pfaltz and Bauer, Waterbury, CT, USA
Cobalt chloride (1.428 mg L^{-1}), $CoCl_3$ x $6H_2O$, CAS 7791-13-1, 100.2%, J.T. Baker, Phillipsburg, NJ, USA
Sodium molybdate (7.26 mg L^{-1}), $Na_2MoO_2 \cdot 2H_2O$, CAS 10102-40-6, 100.6%, J.T. Baker, Phillipsburg, NJ, USA
Copper chloride (0.012 mg L^{-1}), $CuCl_2 \cdot 2H_2O$, CAS 10125-13-0, >99%, SIGMA-ALDRICH, St. Louis, MO, USA
Disodium ethylenedinitrilo tetraacetic acid (300 mg L^{-1}), $Na_2EDTA \cdot 2H_2O$, CAS 6381-92- 6, 100.8%, Fisher Scientific, Pittsburgh, PA, USA
Sodium selenate (2.392 mg L^{-1}), Na_2SeO_4, CAS 13410-01-0, 95%, SIGMA-

ALDRICH, St. Louis, MO, USA

Fish and Ceriodaphnia media (USEPA 1994) for aquatic toxicity tests were prepared by diluting well-water to 90 mg/L total hardness using distilled ASTM Type I water. The pH was adjusted as needed to within the range pH 6.5-7.5. One mL of Cerophyl extract was prepared by adding 3.75 g Cerophyl (Aldon Corp, Avon, NY, USA) to 500 mL distilled ASTM Type I water and mixing in a blender at high speed for 5 min, then allowing to settle overnight in the refrigerator. The supernatant was decanted and divided into 40 mL aliquots and stored frozen, until thawed and used as fish or ceriodaphnia media for aquatic toxicity tests.

5.3 Preparation of Soil

Studies were performed separately and independently for CL-20 in freshly amended soil, and for amended CL-20 that was weathered and aged in soil, in order to determine toxicity benchmark values for CL-20-contaminated soil of each exposure type. Individual CL-20 treatment levels in SSL soil were prepared as single batches for toxicity studies. Each soil batch containing a specific CL-20 treatment concentration was the source of the exposure substrate for definitive tests of CL-20 freshly amended to SSL, substrate for weathering and aging CL-20 in SSL, and each was analyzed to determine CL-20 concentration at the time of introducing test species. During treatment batch preparation, CL-20 was mixed into soil using an organic solvent as a carrier, necessary to dissolve the nonpolar chemical in order to yield a more homogeneous mixture than direct addition of solid chemical crystals to soil. CL-20 was dissolved into acetone and pipetted onto a 2.5 cm thick layer of soil to establish an initial soil concentrate. The acetone was allowed to volatilize for a minimum of 18 h. Photolysis of CL-20 was controlled by volatilizing the acetone in a dark chemical hood. Amended soil was transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 hours on a three-dimensional rotary soil mixer. The final treatment batches were prepared by mixing initially-prepared soil concentrate of CL-20 with clean SSL soil in test-required proportions for 18 hours on a three-dimensional rotary mixer. Carrier control soils were treated with acetone only. After mixing, soil was hydrated with ASTM Type I water to a test-required percent of the soil water holding capacity (WHC; 18% water, on the basis of the dry SSL soil mass, DM) for toxicity testing of CL-20 freshly amended to SSL, or 60% of the WHC for the weathering and aging procedure. Hydrated soils prepared for toxicity tests were allowed to equilibrate for 24 h before exposing test organisms.

Weathering and aging of CL-20 in soil was conducted according to procedures described by Kuperman et al. (2003; 2004; 2005; 2006a,b,c). These procedures included exposing treated and control soil batches, initially hydrated to 60% of the WHC, in open glass containers in the greenhouse at ambient temperature to alternating wetting and drying cycles for twenty weeks. Concentrations of CL-20 in treatment soil batches with nominal concentrations 0.5, 1, 10, and 100 mg kg^{-1} were analytically determined after 1, 6, 8, 15, 17, and 20 weeks of weathering and aging process to determine when the rate of change in CL-20 concentrations substantially declined. This time of weathering and aging duration was then designated for terminating weathering and aging procedures, and for commencement of definitive toxicity testing. During the weathering and aging procedure all soil treatments were weighed and

readjusted to their initial mass at 60% of WHC by adding ASTM Type I water each week to the soil. All soil treatments were brought to a test-required percent of the soil WHC 24 h prior to commencement of toxicity tests.

5.4 Aquatic Test Media

5.4.1 Direct Amendment into Water

The chronic toxicity of CL-20 to ceriodaphnia, and fathead minnow, in directly-amended aquatic media was investigated by using serial dilution of CL-20 stock solution to produce target exposure concentrations. A flask with stock solution of CL-20 (prepared using fish/ceriodaphnia media) was agitated and placed into a sonic water bath for approximately 8 minutes. Ultrasonic treatment created a uniform suspension. The stock solution was then filtered through a 0.45-micron filter and diluted with media to appropriate concentrations. For the algae toxicity assays, a stock solution of CL-20 was prepared using algae media and diluted with unamended media to the appropriate CL-20 concentrations without filtration.

5.4.2 Modified Toxicity Characteristic Leachate Procedure

Soil samples were extracted using a modified Toxicity Characteristic Leaching Procedure (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311) for conducting aquatic toxicity tests with soil elutriates. The modification involved substitution of well-water (for conducting aquatic toxicity tests) in place of acetic acid solution. Prior to extraction, soil samples were moisture-equilibrated in the dark for 24 h at room temperature after addition of well-water. Aquatic toxicity tests using extracts produced from SSL soil amended with CL-20 were prepared by adding 625 g of amended soil to 2500 mL of modified TCLP solution in 1-gallon glass containers. The glass containers were placed into an end-over-end mixer for 18 h. After thorough mixing, the contents of the glass containers were allowed to settle for 1 h. The supernatant was decanted and centrifuged for 20 minutes at 5000 rpm (4420 x G) to clear the suspended material enough to be able to see the organisms during testing. The resulting soil extracts were bubbled with air to raise the pH and dissolved oxygen (DO) to appropriate levels (pH 6.5-7.5 and $\geq$4.0 mg L^{-1} DO, respectively). Measurements of pH were taken using an Accumet Portable pH Meter, Model AP62, and an Accumet Combination Electrode, Model 13-620-AP50; Fisher Scientific, Inc., Hampton, NH, USA. DO measurements were taken using a YSI Oxygen Meter, Model 54A, and YSI Electrode, Model 5739, with Standard Membrane Kit, Model 5775; YSI Incorporated, Yellow Springs, OH, USA. Extracts were then stored in the dark at 4°C. The extracts used in toxicity testing were prepared from SSL soils amended with nominal CL-20 concentrations of 5, 7.5, 10, 20, 30, 40, 50, and 100 mg kg^{-1} without dilution.

Separate SSL soil batches amended with 100 or 1000 mg kg^{-1} nominal CL-20 concentration were subjected to weathering and aging procedure for 482 days as described above. At the end of the weathering and aging process, the soil was extracted using modified TCLP described above to produce modified TCLP solution for aquatic toxicity tests.

5.5 Chemical Extractions and Analyses

Soil samples for analysis were taken after the 24-h moisture equilibration, at the beginning of each definitive test. From each treatment soil batch, 2.3 g of hydrated soil was weighed in triplicate into 50-mL polypropylene centrifuge tubes, 10 mL acetonitrile was added to each tube and the samples were vortexed for 1 min, then sonicated in the dark for 18 hours at 20±2°C. Sonicated samples were centrifuged at 2700 rpm (500xG) for 30 minutes. Five mL of supernatant was transferred to a 20-mL glass vial and combined with 5 mL of $CaCl_2/NaHSO_4$ aqueous solution (5 and 0.2 g L^{-1}, respectively). The samples were shaken and left to equilibrate and settle for 30 minutes. The supernatant was filtered using disposable syringes (10 mL) and 0.45-µm Millipore polytetrafluoroethylene (PTFE) syringe filters. The first 3 mL of filtrate was discarded, and the remainder was retained in a PTFE-capped 4-mL vial. One mL of this filtered solution was transferred to a HPLC vial. The filtered samples were stored in the refrigerator at 4±1°C in the dark no longer than five days if not analyzed on the same day. Soil extracts were analyzed and quantified using a modified EPA Method 8330A (USEPA 1998). The results of acetonitrile soil extractions are reported as CL-20 concentrations in dry soil (105-110ºC oven-dried soil basis). For aquatic toxicity testing, 5 mL of the sample of test media were combined with 5 mL of acidified acetonitrile and processed as above.

In addition to acetonitrile extraction, soil samples collected from the Controlled Environment Soil-core Microcosm Unit (CESMU) columns used in the transport and fate study were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP; Haley et al. 1993). The ATCLP is based on modification of the Toxicity Characteristic Leaching Procedure (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid solution, better simulating field soil-water conditions due to respiration by soil biota. All analytical measurements were done in triplicate at the time of column harvest. For each treatment concentration, 4 g of soil were transferred into 20 mL vials. Sixteen mL of CO_2-saturated water at pH 4.0 was added to the vials, and vials were rapidly sealed tight. Soil samples in CO_2-saturated water were vortexed 45 sec, then leached in the dark for 18 h using an end-over-end mixer rotating at 30 rpm (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311) at room temperature. Soil solids were then allowed to settle, and supernatants were filtered through 0.45 µm syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis.

Concentrations of CL-20 in the soil extracts were determined using an HPLC-UV system, which consisted of an Agilent 1100 HPLC Series equipped with a Supelcosil LC-CN column (25 cm x 4.6 mm x 5 µm), employing an isocratic 70:30 methanol:water mobile phase with a flow rate of 1.0 mL min^{-1} and a 50 µL injection volume. The autosampler was set at 10°C. Blanks and standards were placed among samples having unknown concentration in order to maintain quality assurance of the samples. Detection of CL-20 was accomplished using a Diode Array Detector (DAD) set at 230 nm (λ_{max}) wavelength. A primary stock solution was prepared at 10,000 mg L^{-1} CL-20 in acetonitrile. Intermediate stock solutions of 50, 20, 2, 0.5, and 0.1 mg L^{-1} CL-20 in acetonitrile were then prepared from the primary stock solution. Calibration standards were made from the intermediate stock solutions with acidified water (sodium

bisulfate) solution (50:50) to yield standards of 25, 10, 1, 0.25, and 0.05 mg L^{-1} CL-20 in acidified acetonitrile. Calibration curves were created (r^2>0.99999) with an instrument limit of detection (LOD) of 0.01 mg l^{-1} (S/N=3). Over five months, the reproducibility of the slope was determined to be 149.0 ±5.0 with a %RSD of 3.4 (n=14). The lowest concentration of CL-20 that could be quantified in freshly amended SSL soil was in the nominal treatment of 0.08 mg kg^{-1}, and was 0.098 mg kg^{-1}. The lowest quantified concentration of CL-20 weathered and aged in SSL soil was in the nominal treatment of 0.1 mg kg^{-1}, corresponding to 0.06 mg kg^{-1}.

5.6 Measurement of Soil pH

The pH values of the test soils were determined at the beginning of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual (USDA 1996). Measurements were made with an Accumet® AR 50 pH Meter (digital LCD display; Fisher Scientific, Inc., Hampton, NH USA) using a pH electrode [Accumet pH/ATC combination electrode with silver/silver chloride reference, ±0.002 accuracy] that was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed and blotted. The slope of the buffer measurements was calculated by the meter. If the slope was not between 90% and 102%, the buffer was renewed and rechecked, and the electrode was replaced when necessary. Five grams of ASTM type I water were added to 5 g soil. The soil slurry was vortexed for 10 seconds every five minutes for 30 min. The soil slurry was then vortexed for 10 seconds, one minute before pH measurement. The electrode was rinsed with ASTM type I water and blotted before each pH measurement. The pH was measured in the soil slurry solution above the soil surface while stirring gently, and the value was recorded when the pH reading stabilized.

5.7 Toxicity Assessments

5.7.1 Plant Toxicity Assays

Toxicity of CL-20 to terrestrial plants was assessed according to protocols of American Society for Testing and Materials (ASTM) standard guide for conducting terrestrial plant toxicity tests (E 1963-98; ASTM, 1998) and USEPA early seedling growth test (712-C-96-347, USEPA 1982). Range-finding tests were performed using *Medicago sativa* L. (alfalfa), and *Echinochloa crusgalli* L. (Japanese millet). Nominal CL-20 concentrations in freshly amended SSL soil were 0, 500, 1000, 5000, 10000, and 20000 mg kg^{-1}. Prior to the addition of test soil, a piece of cheesecloth was placed inside each pot at the bottom in order to prevent soil loss during testing. Alfalfa seeds were inoculated with nitrogen-fixing bacteria prior to sowing (Southern States Alfalfa-Clover Nitrogen Fixing Bacteria, lot no. 3092002, expiration date 07/2004 [Alfalfa toxicity tests were completed in 2004]; Southern States Cooperative, Richmond, VA, USA). For each plant species test, twenty seeds of the test species were sown per 10-cm plant pot containing 200 g air-dried soil. Thirty-six mL of ASTM type I water was added to each pot to obtain 100% of the WHC. Plant toxicity tests were performed in a temperature and light controlled growth chamber. The growth chamber conditions were set as follows: light intensity of photosynthetically active radiation (PAR) at 302±15 (SE) μM m^{-2} sec^{-1} (23,094 ±1,197 lux)

with temperature at 25±1ºC for 16 h, and dark exposure at 20±1ºC for 8 h. Plant measurement endpoints included seedling emergence, fresh shoot mass, and dry shoot mass. Dry shoot mass was obtained after drying at 70ºC for 24±2 h. Based on the results of range-finding tests, definitive Limit Tests were performed with alfalfa, Japanese millet, and *Lolium perenne* L. (perennial ryegrass) using a single nominal CL-20 treatment concentration of 10,000 mg kg^{-1}, and appropriate controls. The numbers of emerged seedlings were counted after 5 and 19 d, and shoot fresh and dry mass were measured after 19 d. The reference toxicant, boric acid, was used as the positive control (ASTM 1998).

5.7.2 Soil Invertebrate Toxicity Assays

5.7.2.1 Earthworms Toxicity Test

The chronic 56-day earthworm reproduction assay was used to assess the effects of CL-20 on earthworm *Eisenia fetida*. The test is an adaptation of an International Standardization Organization (ISO) bioassay, ISO 11268-2:1998 *Soil Quality – Effects of Pollutants on Earthworms (Eisenia fetida) – Part 2: Determination of Effects on Reproduction* (ISO 1998a) that is based on test developed by Van Gestel *et al*. (1989). Guidelines for this assay were originally developed for use with Artificial Soil (USEPA Standard Artificial Soil), however research in our laboratory has shown that this assay can also be successfully conducted using natural soils (Simini et al. 2003). The measurement endpoints of this test included number of juveniles produced, number of cocoons produced, and adult survival.

Earthworms were bred in plastic containers filled with approximately 14 kg of a 1:1 mixture of sphagnum PRO-GRO peat moss (Gulf Island Peat Moss Co., PEI, Canada) and BACCTO® potting soil (Michigan Peat Co., Houston, TX, USA). The pH was adjusted to 6.2±0.1 by adding calcium carbonate (pulverized lime). The culture was kept moist at 21±2°C with continuous light**.** Earthworm colonies were fed biweekly with dehydrated alfalfa pellets (27% fiber, 17% protein, 1.5% fat; OB of PA, York, PA) that were fermented, dried, and ground to a course powder. Cultures were synchronized so that all worms used in each test were approximately the same age. Adult worms, 0.3 to 0.6 g, with fully developed clitella were used for testing. Earthworms were acclimated for 48h in the test soil. Earthworms were selected for uniformity and placed on moist filter paper overnight to purge gut contents. Five worms were rinsed twice with ASTM type I water, blotted on paper towels, weighed on an analytical balance, and placed on the soil surface in each of four 400-mL (9 cm diameter), glass jars. The worms were selected randomly for placement across treatments. A 2 g bolus of alfalfa food was added to each jar, moistened with an atomizer, and covered with the soil in the jar. Clear plastic film was stretched over the top of the containers and secured with the screw-on rings, allowing exposure to light. Three small holes were then made in the plastic film with a push-pin to allow for air exchange. Earthworm treatments were incubated under a 16 h light, 8 h dark photoperiod with a mean PAR light intensity of 12.8±0.7 (SE) µM m^{-2} sec^{-1} (985±52 lux) and mean temperature of 21.6±0.1ºC.

After 21 days in the range-finding test, and 28 days in the definitive test, worms were removed from the containers with blunt forceps. The number of surviving earthworms in each

treatment replicate were counted and recorded. Cocoons were counted after 21 days in the range-finding tests as described below. In the definitive tests, worm food was added and clear plastic film and screw rings were placed on the containers as described above. After 28 additional days, cocoons and juveniles in each treatment replicate were harvested, counted, and recorded. Juveniles were induced to crawl to the soil surface by immersing the containers to a level just below the soil surface in a heated waterbath at 41-43ºC for 20-25 min. Juveniles were removed from the soil surface with a blunt forceps and counted. Soil was then spread and examined under a 2.25x lighted magnifier to recover and count any additional juveniles. The total number of juveniles in each container was then recorded. Cocoons were recovered by gently agitating the soil on a 1-mm sieve with water until only the cocoons remained on the surface of the sieve. Cocoons were placed in water in a clear glass dish. Cocoons that floated were counted as hatched; those that sank were counted as unhatched. Cocoons were then examined under the magnifier to confirm whether they had hatched or not. The numbers of hatched, unhatched, and total cocoons per container (treatment replicate) were counted and recorded.

Definitive tests included negative control (no chemicals added), carrier (acetone) control, and positive control. Positive control was prepared as solution of beryllium sulfate in ASTM Type I water using 77 mg kg^{-1} Be nominal concentration. Validity criteria included the following performance parameters for the negative controls:

1) The mean mortality does not exceed 10% in range-finding, and definitive tests;
2) The number of juveniles per five worms is ≥15; and
3) The coefficient of variation for the control reproduction is ≤30% at the end of the test.

5.7.2.2 Enchytraeid Toxicity Test

Definitive tests were conducted both with freshly amended soil, and with CL-20 weathered and aged in SSL soil. Nominal concentrations (mg kg^{-1}) ranged from 0.08 to 10 in tests with freshly amended soil, and from 0.1 to 10 in tests with CL-20 weathered and aged in soil. Definitive tests included negative control (no chemicals added), carrier (acetone) control, and positive control. The positive controls were prepared using a solution of beryllium sulfate in ASTM Type I water in order to prepare nominal concentrations of 45 mg kg^{-1} Be in soil.

The Enchytraeid Toxicity Test was used to assess the effects of CL-20 on the adult survival, and reproduction, of the enchytraeid worm *Enchytraeus crypticus*. The test is an adaptation of an International Standardization Organization (ISO) bioassay ISO/16387 (ISO 2005), which was modified for use with natural soils as described in Kuperman et al. (2003, 2004, 2005, 2006d). The modifications included: (i) different soil hydration levels (described above) required for SSL soil, that has lower WHC compared with Standard Artificial soil, and (ii) shorter test duration for *E. crypticus* potworm (28 d vs. 42 d) due to shorter generation time of the *E. crypticus* species, compared with *E. albidus* potworm for which ISO 16387 test conditions were originally optimized. Tests were conducted using glass jars (42 mm ID; 45 mm deep) containing twenty grams of prepared soil, to which 0.05 g of ground oats were added as food for potworms. Each of the treatments and controls was replicated four times. Adult potworms with eggs in the clitellum region were collected as test candidates from culture maintained in the same SSL soil type. Ten enchytraeid worms selected for uniformity

(approximately 1 cm in length) were placed on top of the prepared soil in each test container. Clear plastic film was stretched over the top of each jar and was perforated with three pinholes to facilitate air exchange. All jars were randomly placed in an environment-controlled incubator at 21.6±0.1ºC and 16:8 h light:dark photoperiod with a mean PAR light intensity of 12.8±0.7 (SE) μM m^{-2} sec^{-1} (985±52 lux) and mean temperature of 21.6±0.1ºC. Individual jars were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM Type I water. Ground oats (0.05 g) were again added to each test container at that time.

Soil in each jar was carefully searched after 14 d to remove and count adult potworms. The remaining test substrate, including any cocoons laid during the first 14 d of the test, was incubated for an additional 14 d. After 28 d from the start of the test, soil in the test jars was fixed with 70% ethanol, and nine drops of Rose Bengal biological stain (1% solution in ethanol) were added. Staining continued for a minimum of 24 h. The contents of each test jar was wet-sieved using a No. 100 mesh (150 μm) sieve, and retained contents transferred to a counting tray where potworms were counted. Measurement endpoints included number of surviving adults after 14 d, and number of juveniles produced after 28 d. All ecotoxicological parameters were determined using measured CL-20 concentration for each treatment level.

Validity criteria were included in the test as part of the Quality Control procedures. They included the following performance parameters for the negative controls:

1) The adult mortality does not exceed 20% after 14 d;
2) The average number of juvenile potworms per test container at the end of the test is greater than 2.5-times the initial number of adult potworms per test container; and
3) The coefficient of variation for the mean number of juveniles is ≤50% at the end of the test.

5.7.2.3 Folsomia Reproduction Test

The Folsomia Reproduction Test (FRT) was used to assess the effects of CL-20 on the reproduction of the collembolan *Folsomia candida*. The test is an adaptation of an ISO bioassay ISO 11267 *Soil quality —Inhibition of Reproduction of Collembola* (*Folsomia candida*) *by Soil Pollutants* (ISO 1998b). The FRT is a chronic assay. The ISO Guideline for this assay was originally developed for use with Artificial Soil (USEPA Standard Artificial Soil). Research in our laboratory has shown that this test can also be conducted using natural soils (Phillips et al 2002; Kuperman et al 2003). The measurement endpoints for the test included juvenile production, and survival of collembola, as adults.

The U.S. Army Edgewood Chemical Biological Center culture of *F. candida* (Collembola) is maintained in culture jars on a mixture of charcoal and plaster of Paris in the dark at nominal 20°C. The Collembola were fed baker's yeast and kept moist by routine misting with ASTM type II water approximately twice per week. Synchronized cultures were established for the experiments by removing egg clusters from stock cultures and placing them into new jars. Eggs were monitored daily to determine the onset of hatching. Once hatching began, it was allowed to proceed for 2 d, after which juveniles were transferred to new jars. These

synchronized juveniles were then held for 10 d, and these procedures provided the 10-12 day-old juveniles used in tests.

Glass test containers (42 mm ID; 45 mm deep) were rinsed successively with acetone, tap water, and ASTM type I water, then inverted and allowed to air-dry. In order to prepare five replicates of each treatment and controls, 100g of each air-dried soil, respectively, was hydrated to 88% of WHC. Then one-fifth of each batch of hydrated treatment soil was transferred by weight into a test container, and 0.05 g of baker's yeast was added to the surface of the soil. Ten 10-12-day-old juveniles were placed in each test container, followed by light misting with ASTM type II water. A piece of clear plastic film was placed on each container, held in place with a rubber band, and the film was then perforated with three pinholes to facilitate air exchange. Thus five replicates were prepared for each CL-20 treatment, plus controls (negative and positive). The mass of each container was then recorded. The test containers were placed randomly in an environment-controlled incubator at 21.6±0.1ºC and 16:8 h light:dark photoperiod with a mean PAR light intensity of 12.8±0.7 (SE) µM m^{-2} sec^{-1} (985±52 lux) and mean temperature of 21.6±0.1ºC. Individual jars were weighed once a week and mass loss was replenished with the appropriate amount of ASTM Type II water.

To terminate a test, approximately 15 mL of tap water was added to a test container and allowed to sit for several minutes to fully hydrate the soil. After gentle mixing with a spatula, an additional 10 mL of water was added. The contents of the test container were then given a final mixing and examined under a dissecting microscope (15x) for the presence of juveniles and adults. Total numbers of juveniles and adults, respectively, that floated to the surface were counted.

Range-finding tests were conducted with freshly amended SSL soils to determine treatment concentrations for the definitive tests. Nominal concentrations used in the range-finding study were: 0, 1, 5, 10, 50, 100, and 500 mg kg^{-1}. Data from the range finding tests were used to select the treatment concentrations for definitive toxicity tests with freshly amended or weathered and aged CL-20 in SSL soil. Analytically determined CL-20 concentrations used in the definitive FRT with freshly amended soil were: 0.25, 0.49, 0.9, 1.0, 5, 10, 25.5, 49, and 97 mg kg^{-1}. Analytically determined concentrations of CL-20 weathered and aged in SSL used in the definitive FRT were: 0.1, 0.3, 0.5, 0.6, 3, 7, 13, 36, and 68 mg kg^{-1}. Definitive tests included negative control (no chemicals added), carrier (acetone) controls, and positive controls. The positive controls were prepared using a solution of beryllium sulfate in ASTM Type I water in order to prepare nominal concentrations of 50 mg kg^{-1} Be in soil. All ecotoxicological parameters in definitive tests were established using measured concentrations of CL-20 in conjunction with the ecotoxicological test results.

Measurement endpoints included total number of juveniles produced, and the number of collembola, surviving as adults, at the end of the 28-d test. Reproduction, and survival of collembola, as adults, in CL-20 treatments were each compared to that of the carrier controls to quantify ecotoxicological parameters. These parameters included the bounded No Observed Effect Concentration (NOEC), the bounded Lowest Observed Effect Concentration (LOEC) and the effective concentration that caused a p-percent reduction (ECp) in the measurement endpoints,

respectively; e.g., EC_{20}, and EC_{50}. Validity criteria were included in the test as part of the Quality Control procedures. These included the following performance parameters for the negative controls:

1) The adult mortality does not exceed 30% after 28-d;
2) The average number of instars is greater than 80 at the end of the 28-d test; and
3) The coefficient of variation for the mean number of instars is ≤30% at the end of the test.

5.7.2.4 Microcosm Assay

SSL soil similar to the one used for establishing ecotoxicological benchmarks for CL-20 in standardized single-species soil invertebrate toxicity tests was used in this study to assess CL-20 effects on the soil microinvertebrate community. During collection of this soil, the root zone of upper soil layer was retained to insure sufficient abundance of the indigenous soil microinvertebrate community. As a result, this soil had slightly different physical and chemical characteristics, including 2.6% organic matter, 14% clay, 58% sand, 28% silt, 9.8 cmol kg^{-1} cation-exchange capacity, and pH 5.1.

Fresh SSL soil containing the indigenous microinvertebrate community was collected in May 2003 from the same location on the property of the U.S. Army Aberdeen Proving Ground, MD, used to collect soil for the single-species soil invertebrate toxicity tests. Soil was gently sieved using a 5-mm sieve to remove large debris and regularize distribution of soil invertebrates. Subsamples (n=5) of prepared soil were extracted immediately to establish the baseline data for abundance of microarthropods and nematodes in SSL soil. This baseline data was used to determine the effects on subsequent soil preparation procedures on the soil microinvertebrate community. Prepared soil was stored in covered plastic containers overnight to preserve the initial field moisture level. Additional SSL soil, collected several days earlier, was defaunated by prolonged heating (three days after constant mass was achieved) at 80ºC, then sieved through a 2-mm sieve, and used to prepare CL-20 soil concentrates. CL-20 soil concentrates were required to uniformly amend fresh field-moist SSL soil during preparation of nominal target treatment concentrations in order to avoid harming soil organisms by exposure to solvent. During the soil concentrate preparation procedure, appropriate amounts of CL-20 were dissolved in 15 g of acetone and pipetted onto measured portions of soil. This approach was used to prepare nominal CL-20 treatments of 100, 500, 1000, and 2500 mg kg^{-1}. The nominal CL-20 treatments of 5000, 7500, and 10000 mg kg^{-1} were prepared by directly mixing appropriate amounts of CL-20 with dry SSL soil because large quantities of CL-20 required for preparation of these treatments could not be dissolved in 15 g of acetone. Acetone was added to these three treatments in the same amount (15 g) to maintain the uniformity of treatments throughout all exposure concentrations. The acetone was allowed to volatilize in a fume hood overnight in the dark. Amended soil batches were then mixed for 18 h using a three-dimensional rotary soil mixer.

Nominal target concentrations for all freshly-amended treatments were prepared one day after collecting soil in the field by individually combining and gently mixing CL-20-amended soil concentrates with clean field soil in a plastic bag. This approach ensured that the amount of fresh SSL soil containing indigenous soil biota remained constant throughout the range of treatments. The carrier control treatment was amended with acetone treated SSL soil

only. The field soil moisture level at the time of soil collection was maintained for the duration of the study by weekly additions of ASTM type I water, in order to adjust to initial treatment weights. The final nominal CL-20 treatment concentrations prepared for definitive test included 0 (carrier control), 100, 500, 1000, 2500, 5000, 7500, and 10000 mg kg^{-1}. Soil samples from each treatment were analyzed by HPLC using a modified USEPA Method 8330A (USEPA 1998) to determine acetonitrile-extractable CL-20 concentrations. Results of analytical determinations of CL-20 in soil treatments showed that the initial CL-20 concentrations were below detection limit (BDL) in carrier control, 108, 479, 870, 2355, 4635, 7020, and 10300 mg kg^{-1}, respectively. Concentrations of CL-20 in all treatments at each harvest date are shown in Table 12.

The duration of the definitive test was 12 weeks, and included three harvests at four-week intervals, and included assessment of the potential effects of weathering and aging of CL-20 in soil on the bioavailability of CL-20 and its toxicity to soil biota. The definitive test was conducted using four replicates per treatment per harvest date (total of 12 replicate test containers per treatment). Approximately 200 g of soil were loosely packed into individual test containers (glass jars 900 mL volume, 90 mm diameter). The total mass of each test container with soil was recorded. Clear plastic film was stretched over the top of each container, secured with a rubber band, and the plastic film was perforated with three pinholes to facilitate air exchange. All containers were randomly placed in an environment-controlled incubator at 22±1°C, 86% relative humidity (RH), 16:8 h light/dark photoperiod cycle. ASTM Type I water was added weekly to maintain the initial soil moisture level.

A set of four randomly selected replicates from within each treatment was processed during the first, second, and third (final) harvests, after the 4-, 8-, and 12-week exposure periods, respectively. Soil was gently mixed inside individual test containers, and then sampled to determine percent moisture, CL-20 concentrations in test soil, and to extract microarthropods and nematodes. Soil microarthropods were extracted into 76% ethyl alcohol using high-gradient extractors (Merchant and Crossley 1970). The most abundant microarthropods were sorted to acarine suborders Prostigmata, Mesostigmata and Oribatida, and the insectan order Collembola (springtails). Miscellaneous groups, including classes Diplopoda and Symphyla, and the insect orders Diplura, Psocoptera (psocids), Thysanóptera (thrips), Coleoptera (beetles) larvae, and Diptera (flies) larvae, were recorded and added to the total number of microarthropods for statistical analyses. Nematodes were extracted using Baermann funnel method (McSorley and Walter 1991) for 48 h at room temperature (Parmelee et al. 1993). Live nematodes were counted at 140x and were sorted into bacterivore, herbivore, fungivore, omnivore, and predator trophic groups, and a group of hatchlings as described in Parmelee et al. (1993). Abundance of soil invertebrates was expressed as number of individuals per g dry soil (ind g^{-1}).

5.7.3 Litter Decomposition Assay

Litter decomposition rates were quantified using Orchard grass (*Dactylus glomerata*) straw collected in grassland on the property of Aberdeen Proving Ground, MD, in May 2003. Orchard grass straw was dried at 60°C until constant mass was achieved, and cut into 5-cm long internodular sections. Three pieces of these straw sections were used to form a straw cluster. The mass of each cluster was recorded and an aluminum tag with an identification number was attached

to each cluster with a nylon string. Pre-weighed clusters of three straw sections of approximately 0.2 g each were placed on the soil surface in each test container used in the microcosm study described above. One cluster of Orchard grass straw was harvested from a set of three randomly selected replicates from within each treatment after 1, 2, 3, 4, 6, and 8 months of exposure, and processed to determine litter mass loss. Harvested clusters of Orchard grass straw were gently rinsed with ASTM Type I water to remove soil debris, then oven dried at 60°C until constant mass was achieved (approximately 24 h).

Mass loss data from each harvest date was used to compare litter decomposition rates in individual CL-20 treatments of SSL soil, ranging from nominal 0 to 10,000 mg kg^{-1}. Annual decay rate constants (k) for litter residues, and corresponding standard errors (SE) and regression coefficients (r^2) were determined using the single negative exponential decay model $m_t/m_0 = e^{-kt}$, where m_t/m_0 = fraction mass remaining at time t, t = time elapsed in years, and k = the annual decay constant. The single negative exponential model was fit to the data by least squares regression of the natural logarithm of mean percent mass remaining over time. Analysis of variance (ANOVA) and Fisher's least significant difference pairwise comparison tests were used to determine the effects of CL-20 on litter decay rate constants at a significance level of $p \leq 0.05$.

5.7.4 Aquatic Toxicity Assays

We selected the three aquatic species for assessing the aquatic toxicity of CL-20, including *Ceriodaphnia dubia* (daphnid), *Selenastrum capricornutum* (green alga), and *Pimephales promelas* (fathead minnow). This selection was based on representative phylogeny, ecological relevance, and the availability of standardized methods that allow the determination of potential acute as well as chronic toxicity for all three species. These aquatic bioassays were designed to provide concentration-response information by assessing the mortality, growth, and reproductive measurement endpoints. All three methods were recommended by the USEPA for testing of effluents, and may be applied to testing of pure compounds (USEPA 1994). All organism stocks were cultured in-house under USEPA guidelines (1994). Toxicity of CL-20 to aquatic species was determined from exposures to both CL-20 in aquatic media, and to elutriates from CL-20 amended SSL soil. The ecotoxicological parameters included IC_{20}, IC_{50} values, and bounded NOEC and LOEC values.

5.7.4.1 Algal Growth Inhibition Assay

Algal Growth Inhibition Assays with directly-amended growth media were conducted to determine the effect of CL-20 on growth of the green alga *Selenastrum capricornutum*. Stock cultures of unicellular *S. capricornutum* were grown in 3-L batch cultures and taken during log phase growth to inoculate test flasks. Stock cultures were periodically checked for bacterial contamination, and eliminated if needed. Culture media were prepared according to standard methods (USEPA 1994) using stock solutions of macro- and micro-nutrients prepared using distilled ASTM Type I water (as described in Section 5.2). Based on cell density measurements of stock algae cultures, the volume necessary to yield an initial cell density of 1×10^4 cell mL^{-1} per test chamber was determined. This volume was added to each treatment group and control, producing a

total volume for each of 100 mL of solution. The treatment groups and control were subjected to test conditions for 10 days (static non-renewal). Following inoculation with algae, individual test chambers were randomly placed in an incubator at 25±1°C, and an average PAR exposure of 44±2 (SE) μM m^{-2} sec^{-1} of continuous light. The vessels were shaken by hand twice daily. Cell density determinations were made via the manual microscope counting method (hemocytometer) at time 96 hours and 10 days. Hardness, conductivity, and pH of test samples were measured prior to commencing algal toxicity testing.

5.7.4.2 Ceriodaphnia Survival and Reproduction Assay

Ceriodaphnia dubia were grown in conditioned well-water that was passed through a micronizer (air injection), limestone bed (pH adjustment), iron removal system, carbon filtration, particulate filtration, and UV sterilization. The well-water was diluted with distilled ASTM Type I water to yield a water hardness of 90 ppm. The organisms were maintained as batch cultures in 800 mL of media. The batch cultures were maintained for 14 days while initiating new cultures every five to seven days. Ceriodaphnia were fed an algal mixture of *Selenastrum capricornutum, Chlamydomonas reinhardti* and cerophyl. The algae were cultured (USEPA 1994) for approximately 7 days before being harvested and fed to the ceriodaphnia at a concentration of 10^6 cells mL^{-1}. The Cerophyl (dehydrated cereal of grass leaves) stock solutions were prepared by suspending 3.75 g of Cerophyl in 500 mL of ASTM Type I water. The mixture was placed into a blender at high speed for 5 minutes. The solution was allowed to stand overnight in a refrigerator (4±1°C) to settle out the large particulate. The supernatant was then decanted and stored frozen in 40 mL aliquots. Thawed Cerophyl supernatant was added to the test media at the proportion of 1mL/100 mL of test media.

Adult ceriodaphnia were isolated from the stock cultures approximately 2 weeks before test initiation. The second generation from the adult daphnids was used for brood stock production. The F_2 brood stock or higher (<24 hours old) was transferred to clean media and used in the Ceriodaphnia Survival and Reproduction Assays.

Ceriodaphnia test chambers consisted of 30 mL plastic beakers containing a total of 15 mL of solution. There were ten replicates of each treatment group and control, containing one ceriodaphnia each. The media was changed and fresh food added daily for 7 days. Mortality, reproduction, pH, and dissolved oxygen measurements were recorded every 24 h. A diurnal light cycle was maintained at 16 h light:8 h dark. The PAR light intensity averaged 10±1 (SE) μM m^{-2} sec^{-1}. The temperature was maintained at 25±1°C. Tests were terminated when 60% of the control organisms had produced their third brood, or at the end of eight days, whichever occurred first.

5.7.4.3 Fathead Minnow Survival and Growth Assay

Water for the fish assays was drawn from a 400-ft deep well. The well-water was conditioned as described for the Ceriodaphnia Survival and Reproduction Assay. Adult fish were maintained in 55-gallon glass aquaria equipped with under-gravel filtration units. Adults were fed twice daily [with *Lumbriculus variegatus* (aquatic black worms) in the mornings, and with

Tetramine Flake Food (Tetra Holding Inc., Blacksburg, VA, USA) in the afternoons]. Water temperature was maintained at 25±1°C with a diurnal light cycle (PAR light intensity average of 10±1 (SE) μM m^{-2} sec^{-1}) of 16 h light:8 h dark. Terra-cotta shards were placed in the aquaria to provide egg-laying substrates. The pots were checked every morning for eggs. If eggs were present, the pots were removed and placed in hatching containers. Larvae were collected within 24 h of hatching and used in assays. The test chambers consisted of 1-L glass jars with screw-top lids, and were filled with 400 mL of solution. Individual test chambers were randomly positioned on the test table, with room temperature and light cycle maintained as described above. The larvae were placed into test chambers using a large bore plastic pipette, and were fed freshly hatched brine shrimp twice daily. Water changes (every 24 h) were done by siphoning out the water and debris using an air-vacuum tube attached to a 1-mL plastic pipette. Approximately 10% of the solution was retained in the jars to allow the larva room to swim freely before the addition of new media. Dissolved oxygen, pH, and mortality were recorded daily up to 7 days. At the conclusion of testing the larva were removed, rinsed in distilled water, and oven-dried at 100°C for 2 h. The dry weights of the larvae were measured to the nearest 0.01 mg.

5.8 Transport and Fate of CL-20 in Soil

Assessment of transport and fate of CL-20 in soil have been determined using an improved system, the Controlled Environment Soil-core Microcosm Unit (CESMU), adapted from the standard soil microcosm design (USEPA 1987; ASTM 2004b). An important modification to the original design was the application of tension at the bottom of each soil column to mimic field conditions and prevent the build-up of water within columns, which can otherwise change the chemical, physical, and biological properties of soil. This modification required placement of a controlled-pore porous ceramic plate at the bottom of intact soil core to further approximate physical environment of undisturbed soil during application of tension.

The same SSL soil type used in toxicity tests was used in transport and fate study. Intact (non-homogenized) soil cores were extracted from the same site using a hydraulically controlled probe delivering soil cores with minimal disturbance directly into inert high-density polyethylene pipe (HDPE). Collection, transportation, and storage of soil core columns were done following procedures described in Checkai *et al*. (1993). All soil columns that passed the throughput test were assembled in controlled environment chambers in the greenhouse. The CL-20 concentrates of 100 and 10,000 mg kg^{-1} in SSL soil were prepared using soil amendment procedures described in Section 5.3. The CL-20 transport and fate study started in April 2004 and was completed in December 2004. During the 35-week study, CL-20 concentrations in soil were periodically determined using acetonitrile extraction method for soil collected from different depths. These determinations were done in the subsets of replicate intact soil core columns, randomly selected and harvested at 7-week intervals. Selected columns were first removed from the CESMU chambers. All ryegrass plants were harvested at the soil level, weighed, and placed in a –80ºC freezer until they were analyzed. Soil columns were sealed and frozen at –4ºC. After 24 h in the freezer, each HDPE pipe (containing a harvested soil-core) was carefully cut length-wise using an electric router, allowing the intact soil-core to rest in the lower half of the pipe. Soil-cores were allowed to come to ambient temperature in the horizontal position to eliminate longitudinal migration. Soil-cores were sectioned incrementally to produce the following soil depth sections: 1 (0-2 cm) containing CL-20 that was initially placed atop each column, or acetone treated control

soil, 2 (2-3 cm), 3 (3-5cm), 4 (5-7 cm), 5 (7-12 cm), 6 (12-22 cm), 7 (22-27 cm), and 8 (below 27 cm). Each soil section was weighed, transferred to a clean plastic bag, and manually homogenized. Sub-samples of soil were removed and prepared for chemical analyses, and dry fraction determinations.

The fate of that portion of CL-20 that partitions into water was assessed by analyzing both the leachates throughout the study period, and the plant tissues of perennial ryegrass *Lolium perenne*. During the study synthetic rainwater was administered to the soil-cores using a peristaltic pump at an application rate of 2.54 cm h^{-1} (1"/h) two times each week for six weeks prior to the scheduled harvests of individual columns, and daily during the last week preceding each harvest in order to allow sufficient quantity of leachate required daily during that period for aquatic toxicity testing with ceriodaphnia. Synthetic rainwater was administered uniformly to individual columns for soil hydration in all treatments, totaling 1.3 L precipitation for each 7-week period. This application rate was based on historic measurements of total-average regional precipitation. Leachates were collected into glass flasks housed in darkness within the controlled environment chamber, and sampled for analysis following each synthetic rainwater application. Analytical determinations of CL-20 in soil sections (by depth) and leachates followed procedures described in Section 5.5.

5.9 Bioaccumulation Potential of CL-20 in Plants

In addition to quantifying CL-20 concentrations in the leachates throughout the study period, the fate of that portion of CL-20 that partitions into water was assessed by analyzing the plant tissues of *Lolium perenne* L. (perennial ryegrass) harvested from the intact soil core columns at the end of the 35-week study. CL-20 concentrations in plant shoot tissues were also used to determine the bioaccumulation potential of CL-20 in *L. perenne*. Ryegrass plants were harvested at the soil level from three replicate columns from each of the control, 100, and 10,000 mg kg^{-1} nominal CL-20 treatments. Ryegrass shoot tissues were weighed and placed into a –80ºC freezer in sealed plastic bags until they were extracted and analyzed. CL-20 was extracted according to procedures (Number LB006.e1, dated 2005) developed by the Biotechnology Research Institute of NRC-Canada. The procedure is summarized as follows: Bags containing plant tissues were removed from freezer, opened slightly, and immediately placed in a lyophilizer (freeze-dryer) for approximately 20 h. Freeze-dried tissue was then ground to pass through a 40 mesh screen using a Wiley mill, then the 0.2 g subsamples of tissue were placed in 50 mL centrifuge tubes. Ten mL of 0.5 μg mL^{-1} HMX (used as internal standard) in acetonitrile (ACN) were then added to each centrifuge tube, and the samples were vortexed for one minute, then placed in a thermostated ultrasonic bath at 20±2°C for 18±2 h. After sonication, samples were centrifuged at 2700 rpm (500 xG) for 30 min. Three mL of supernatant were removed and combined with three mL of $NaHSO_4$ aqueous solution (0.2 g L^{-1}) in a 20-mL vial. The samples were vortexed for approximately 10 seconds, then left to stand for 30 min. The supernatants were filtered using disposable syringes (10 mL) and 0.45 μm-Millipore PTFE syringe filters. The first 3 mL of filtrate was discarded, and the remainder was retained in a PTFE-capped 4-mL vial. One mL of the filtered solution was transferred to an HPLC vial. The filtered samples were stored in the refrigerator at 4°C if not analyzed the same day. Analytical determinations of CL-20 in plant tissues followed procedures described in Section 5.5.

5.10 Data Analyses

Linear or nonlinear regression analyses to derive ecotoxicological benchmark values were conducted on untransformed data from standardized single-species toxicity tests based on the concentration-response relationships for quantitative endpoint data using regression models described in Stephenson et al. (2000). Survival data from the microcosm assay were normalized using square root (x+1) transformation prior to using in regression analyses; the exception was predatory nematode survival data from the second harvest that did not require transformation. The ECp (effective concentration for a specified percent effect) values for cocoon/juvenile production and adult survival (when appropriate) in the soil invertebrate tests, or the ICp (inhibiting concentration for a specified percent effect) values in the aquatic toxicity tests, were determined using SYSTAT 7.0 (SPSS Inc., 1997) or SYSTAT 11 (Systat Software Inc**.** (SSI), Richmond, California, USA, 2004). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether to weight the data, and in order to select potential models. The following nonlinear regression models were considered.

Exponential model: $Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp})^{\times} C) + b$ (1)

Logistic Gompertz model: $Y = a \times e([\log(1\text{-}p)]^{\times} [C/\mathrm{ECp}]^{b})$ (2)

Logistic Hormetic model: $Y = (t \times [1 + hC] / \{1 + [(p + h\ \mathrm{ECp}) / (1 - p)] \times [C/\mathrm{ECp}]^{b}\}$ (3)

where

Y	=	number for a measurement endpoint (e.g., number of juveniles)
a	=	control response
t	=	control response in the hormetic model
e	=	base of the natural logarithm
p	=	percent inhibition/100 (e.g., 0.50 for EC_{50})
C	=	exposure concentration in test soil
ECp	=	estimate of effect concentration for a specified percent effect
h	=	hormetic effect parameter
b	=	scale parameter

The ECp parameters used in this study included the CL-20 concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameters based on reproduction or growth endpoints are the preferred parameters for deriving Eco-SSL values. The EC_{50} parameters, a commonly reported value, were included to enable comparisons of the results produced in these studies with results reported by other researchers. Similarly, the ICp parameters used in aquatic toxicity assays included the CL-20 concentration producing a 20% (IC_{20}) or 50% (IC_{50}) inhibition in quantitative endpoint data. The IC_{20} parameter based on growth or reproduction endpoints were included to determine conservative/safe level for use in Ecological Risk Assessment. The IC_{50} was included to enable comparisons of the study results with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values. The effects on earthworm growth were estimated using Analysis of Covariance (ANCOVA) by determining the least square mean adult-final live mass per worm, adjusted for the covariate, initial live mass per earthworm. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. When NOAEC (no observed adverse effect concentration) or LOAEC (lowest observed adverse effect concentration) values were determined, the same statistical methods were used. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values. Student's *t*-Test (two-tailed) with significance level set at $p \leq 0.05$ was used in the Limit Tests, and to analyze the baseline and control data for microinvertebrate groups in the microcosm assay, using EXCEL software (Microsoft Corporation, 1997). All analyses were done using measured CL-20 concentrations.

6. Results and Accomplishments

6.1 Effect of CL-20 on Terrestrial Plants

Toxicity tests were performed to determine the effect of CL-20 on growth and seedling emergence of three plant species, *Medicago sativa* L. (alfalfa), *Echinochloa crusgalli* L. (Japanese millet), and *Lolium perenne* L. (perennial ryegrass). Results from range-finding tests in this study showed that CL-20 did not significantly ($p>0.05$) reduce seedling emergence or shoot growth of alfalfa or Japanese millet up to 20,000 mg kg^{-1}. Therefore, the Limit Tests were performed using the three plant species. Treatment levels consisted of carrier control, nominal 10,000 mg kg^{-1} CL-20 in dry soil, and a positive control (boric acid). SSL soil was amended with dry crystalline CL-20 and mixed in a three dimensional mixer for 24 h. Soil was then hydrated to 10.8% moisture (w/w dry soil basis), which is equal to 60% of the WHC of SSL, and both control and amended soils were placed in separate 28 cm x 38 cm (5 L) glass pans in a greenhouse. Weathering and aging of CL-20 in soil was simulated by allowing the soil to dry in cycles, rehydrating once each week for 16 weeks. The soil surface was lightly mixed with a spatula before hydrating to avoid formation of surface crust. Following the weathering and aging process, soil was mixed with a spatula and divided into three equal parts for tests with each of the three plant species.

Extraction of weathered and aged CL-20 from soil with acetonitrile yielded 7,856±126 (SE) mg kg^{-1} CL-20 at the start of the toxicity tests. Results of the plant toxicity tests are shown in Table 2. Mean dry mass, fresh mass, and seedling emergence of all three species grown in soils amended with CL-20 were not significantly different ($p>0.05$) from those grown in carrier control soils according to Student's *t*-test (Table 2). Mean fresh mass and dry mass of alfalfa shoots grown in soil amended with CL-20 were 0.598 and 0.175 g, respectively; and mean fresh mass and dry mass of alfalfa shoots grown in carrier control soil were 0.428 and 0.129 g, respectively. Mean fresh mass and dry mass of Japanese millet shoots grown in soil amended with CL-20 were 2.10 and 0.373 g, respectively; and mean fresh mass and dry mass of Japanese millet shoots grown in carrier control soil were 1.76 and 0.302 g, respectively. Mean fresh mass and dry mass of perennial ryegrass shoots grown in soil amended with CL-20 were

0.894 and 0.196 g, respectively; and mean fresh mass and dry mass of perennial ryegrass shoots grown in carrier control soil were 1.020 and 0.194 g, respectively. Shoot fresh mass of alfalfa, Japanese millet, and perennial ryegrass grown in soil amended with boric acid, the positive control, was reduced by 7, 12, and 50%, respectively, and all were significantly different ($p \leq 0.05$) compared with carrier control. Shoot dry mass of alfalfa, Japanese millet, and perennial ryegrass grown in soil amended with boric acid was reduced by 18, 25, and 54%, respectively, and all were significantly different ($p \leq 0.05$) compared with carrier control.

Mean number of alfalfa seedlings emerged in soil amended with CL-20 or carrier control soil was 14.1 and 14.3 g, respectively; mean number of Japanese millet seedlings emerged in soil amended with CL-20 or carrier control soil was 18.9 and 18.5 g, respectively; and mean number of perennial rye seedlings emerged in soil amended with CL-20 or carrier control soil was 18.9 and 19.3 g, respectively. Seedling emergence of alfalfa, Japanese millet, and perennial ryegrass grown in soil amended with boric acid was 106, 95, and 95%, respectively, compared with carrier control.

Table 2. Mean fresh and dry mass, and seedling emergence (n = 4; 20 plants per replicate) of alfalfa, Japanese millet, and perennial ryegrass exposed to CL-20 in Sassafras sandy loam soil.

Test Species	CL-20[a] (mg kg^{-1})	Fresh mass (g)	SE[b]	Dry mass (g)	SE	Seedling emergence (count)	SE
Alfalfa	0	0.43	0.09	0.13	0.03	14.1	0.7
	7,856	0.59	0.06	0.18	0.01	14.3	0.5
p[c]		0.13		0.13		0.11	
Japanese millet	0	1.76	0.17	0.30	0.03	18.5	0.4
	7,856	2.10	0.16	0.37	0.03	18.9	0.5
p		0.16		0.12		0.58	
Ryegrass	0	1.02	0.05	0.19	0.01	19.3	0.25
	7,856	0.89	0.08	0.20	0.01	18.9	0.35
p		0.21		0.88		0.58	

Table notes:
[a] Acetonitrile extraction and analysis using USEPA Method 8330A
[b] SE = Standard Error of the mean.
[c] *p*-value derived from two-tailed Student's *t*-test (SYSTAT® 11.0).

Results of our studies showed that CL-20 did not adversely affect growth or seedling emergence of *Medicago sativa* L. (alfalfa), *Echinochloa crusgalli* L. (Japanese millet), and *Lolium perenne* L. (perennial ryegrass) in SSL soil producing the unbounded NOEC value of 7,856 mg kg^{-1}. These results were obtained in a soil that supports relatively high bioavailability of CL-20, with species that represent a wide variety of plant genera and habitats. Alfalfa is a dicotyledonous species. Perennial ryegrass and Japanese millet are monocotyledonous species. Alfalfa and perennial ryegrass are crop plants, whereas Japanese millet grows naturally and can tolerate relatively wet habitats. Furthermore, another study with two of the same species in the same soil type produced similar results. Gong et al. (2004) reported no adverse effect of CL-20 on alfalfa and ryegrass up to nominal 10,000 mg kg^{-1} in SSL soil in similarly designed studies. Interestingly, in those studies ryegrass shoot biomass in CL-20 treated soils was significantly greater when compared with control soils, although the effect was not concentration dependent. Those authors reported that up to 200 mg kg^{-1} CL-20 was found in plant tissue, and that treated soils had nearly 20% less CL-20 at the end of the test than when the test was initiated. Those authors reported that possible pathways included plant uptake, biodegradation (e.g., nitrate reductase activity), hydrolysis, or binding to soil particles. On the basis of our plant toxicity test results, plus those of Gong et al. (2004), we conclude that CL-20 was relatively non-toxic to the representative plant species tested, and therefore is also expected to be relatively non-toxic to the majority of terrestrial plants.

6.2 Effects of CL-20 on Soil Invertebrates

6.2.1 Analytical Determinations of CL-20 in Soils Used in Single-species Toxicity Tests

Concentrations of CL-20 decreased in monitored 0.5, 1, and 10 mg kg^{-1} treatments during a 20-week weathering and aging procedure. The average (± standard error) percent recoveries of CL-20 among the three monitored treatments were 97±3, 88±3, 75±2, 76±3, and 70±3% of the initial concentrations after 6, 8, 15, 17, and 20 weeks, respectively, of weathering and aging. Analytical determinations 20 weeks after initiation of the weathering and aging procedure showed that the rate of change in CL-20 concentrations had substantially declined in treatments representing low, intermediate, and high levels of the exposure range selected for definitive testing. The definitive testing for CL-20 weathered and aged in SSL was initiated with soils having approximately 60% of initial concentrations.

Soil pH (±0.02) in negative and carrier controls was 5.09 and 5.65, respectively, in freshly amended treatments, and 5.38 and 5.40, respectively in weathered and aged treatments. Soil pH in SSL freshly amended with CL-20 ranged from 5.47 to 5.67. Soil pH in SSL amended with CL-20 and subjected to a 20-week weathering and aging process ranged from 5.31 to 5.40. There was no relationship between concentration of CL-20 and soil pH in either freshly amended or weathered and aged CL-20 treatments.

Analytically determined CL-20 concentrations in freshly amended soils used in definitive toxicity tests averaged 105 percent (range from 97 to 123%) of nominal concentrations (Table 3), indicating good correlation between the nominal and measured CL-20 concentrations determined in our study after a 24-h equilibration period for soils hydrated to 60% of the WHC. This percent recovery was comparable with an average of 87% recovery determined by Robidoux et al. (2004) in their study with similar SSL soil immediately extracted following the amendment with nominal CL-20 concentrations ranging from 0.32 to 10 mg kg^{-1}. Overall, our results of chemical analyses confirmed that the soil amendment procedure used in toxicity tests was appropriate, and that the USEPA Method 8330A was efficient for quantifying the amount of CL-20 in soil.

Special consideration in assessing CL-20 toxicity was given to the effects of weathering and aging of CL-20 in soil on the exposure of soil receptors. Assessment of the CL-20 toxicity included studies with CL-20 weathered and aged in amended soils to more closely simulate the exposure effects in the field locations, where CL-20 may persist for extended periods of time. CL-20 concentrations in soil decreased 41%, on average, in response to weathering and aging, with individual recovery percentages ranging from 44 to 74%, compared with initial concentrations in freshly amended soils (Table 3). Primary efforts of this phase of investigation were focused on establishing the net toxic effects on the soil invertebrate species tested from exposure to contaminated soil that may be attributed to the presence of CL-20 in an aerobic soil environment; hence identification of the breakdown products of CL-20 weathered and aged in soils was not included in analytical determinations. Overall, chemical analyses demonstrated that chemical exposures for soil invertebrate in soils amended with CL-20 and subjected to weathering and aging differed from those of soils containing freshly amended CL-20. The differing exposure conditions included decreased concentrations of CL-20, potential

presence of any degradation products of CL-20 that could be formed during weathering and aging (Balakrishnan et al. 2003, 2004a, b; Trott et al. 2003), and potential alteration of CL-20 bioavailability in soil to the test species. The inclusion of the weathering and aging component in the toxicity assessments allowed us to incorporate potential alterations in the soil chemical environment, and corresponding changes in toxicity at contaminated sites, into the development of toxicological benchmarks for soil invertebrates.

Table 3. Recovery of CL-20 from Sassafras sandy loam soil in freshly amended, and weathered and aged treatments used in definitive toxicity tests with soil invertebrates.

Freshly amended treatments			Weathered and aged treatments	
Nominal	Determined Initial	Recovery	Determined Final	Final/Initial
mg kg^{-1}	mg kg^{-1}	%	mg kg^{-1}	%
0 (NC)	BDL[a]		BDL	
0 (AC)	BDL		BDL	
0.01	BDL		BDL	
0.02	BDL		BDL	
0.03	BDL		BDL	
0.06	BDL		BDL	
0.08	0.098 (0.003)	123	BDL	
0.1	0.112 (0.003)	112	0.061 (0.002)	54
0.25	0.271 (0.004)	108	0.12 (0.03)	44
0.5	0.487 (0.031)	97	0.28 (0.01)	58
0.75	0.901 (0.033)	120	0.48 (0.01)	53
1	1.005 (0.008)	100	0.61 (0.02)	61
5	4.99 (0.09)	100	3.10 (0.01)	62
10	10.1 (0.4)	101	6.76 (0.14)	67
25	25.5 (1.21)	102	12.5 (0.08)	49
50	48.9 (0.72)	98	36.3 (1.65)	74
100	97.2 (1.27)	97	67.5 (0.32)	69

Table notes: Concentrations are based on acetonitrile extraction and HPLC using USEPA Method 8330A. Values are means (n=3) and standard errors (SE). Initial concentrations were determined after a 24-h equilibration period for soils hydrated to 60% of the soil water holding capacity. Final CL-20 concentrations were determined after a 20-week weathering and aging process. [a] BDL = below detection limit; limit of detection was 0.098 mg kg^{-1} dry soil in freshly amended treatments, and 0.06 mg kg^{-1} in weathered and aged treatments. NC = negative control. AC = acetone control.

6.2.2 Effects of CL-20 on Earthworm *Eisenia fetida*

Earthworm Range-Finding Toxicity Test

Earthworms were exposed to nominal CL-20 concentrations of 0, 1, 5, 10, 50, 100, and 500 mg kg^{-1} in soil. No cocoons were produced at CL-20 concentrations of 1.0 mg kg^{-1} and above in the range finding study. Adults were smaller, more rigid, and more lethargic in all CL-20 treated soils compared to those in control soils. However, adult survival was not significantly (p>0.05) reduced at any level of CL-20 compared to controls.

Earthworm Definitive Toxicity Tests

Treatment concentrations were determined using the results of the range-finding studies. Nominal concentrations (mg kg^{-1}) of freshly amended CL-20 in soils used in the definitive earthworm toxicity tests were: 0, 0.01, 0.02, 0.03, 0.06, 0.08, 0.10, 0.25, 0.50, 0.75, 1.0, and 5.0. Nominal concentrations (mg kg^{-1}) of weathered and aged CL-20 in soils used in the definitive earthworm toxicity tests included: 0.01, 0.03, 0.06, 0.08, 0.10, 0.25, 0.5, 0.75, 1.0, 5.0, and 10. Test results complied with validity criteria defined in the ISO 11268-2:1998 test guideline (Section 5.7.2.1). Mean adult survival in negative controls was ≥90% in all tests. Juvenile production in positive controls ranged from 54 to 98 percent reduction compared with negative controls and was within the baseline established for the laboratory culture of *E. fetida*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to CL-20 treatments.

Results of the definitive earthworm toxicity tests showed that both freshly amended and weathered and aged CL-20 decreased both reproductive capacity and growth of the earthworm, *Eisenia fetida* in SSL soil (Tables 4 and 5). Ecotoxicological responses of *E. fetida* to freshly amended CL-20 in SSL soils are shown in Table 4. In these soils, the numbers of total and hatched cocoons were significantly (p≤0.0002) reduced at measured soil concentrations of CL-20 of 0.11 mg kg^{-1} (nominal 0.1 mg kg^{-1}) and above. Juveniles were significantly (p≤0.0002) reduced at 0.27 mg kg^{-1} (nominal 0.25 mg kg^{-1}) and above. Adult live mass and juveniles per hatched cocoon were reduced at or above 1.0 mg kg^{-1} (nominal 1.0 mg kg^{-1}; Table 4). Adult survival of *E. fetida* in freshly amended soils was not affected by CL-20 concentrations up to and including 5 mg kg^{-1}. Weathering and aging of CL-20 in SSL soil increased the effective toxicity of CL-20 in soil to earthworms. In weathered and aged soils, the numbers of both total and hatched cocoons were significantly (p≤0.001) reduced at nominal soil concentrations of CL-20 of 0.03 mg kg^{-1} and above. Juveniles were significantly (p≤0.0002) reduced at nominal 0.08 mg kg^{-1} and above. Juveniles per hatched cocoon were significantly (p≤0.001) reduced at or above measured concentrations of 0.48 mg kg^{-1} (nominal 0.75 mg kg^{-1}; Table 4). Adult live mass per worm was reduced at or above 3.10 mg kg^{-1} (nominal 5.0 mg kg^{-1}; Table 4). Adult survival of *E. fetida* in weathered and aged soils was not affected by measured CL-20 concentrations up to and including 6.76 mg kg^{-1} (nominal 10.0 mg kg^{-1}).

The concentration-response relationships for CL-20 versus cocoon and juvenile production, determined by nonlinear regression analyses, are shown in Figure 2 (freshly amended CL-20) and Figure 3 (weathered and aged CL-20). The exponential model was the best fit for the data resulting from studies utilizing freshly amended CL-20 in soil (Figure 2). In the studies utilizing CL-20 weathered and aged in soil, the Gompertz logistic model had the best fit

for the earthworm toxicity data. Ecotoxicological benchmarks for both freshly amended and weathered and aged CL-20 treatments that represent NOEC, LOEC, EC_{20}, and EC_{50} values, respectively, for number of earthworm cocoons, juveniles, and adult survivors are presented in Table 6. Bounded NOEC and LOEC values for cocoon production were 0.098 and 0.11 mg kg^{-1}, respectively, based on measured values of freshly amended CL-20. Bounded NOEC and LOEC values for juvenile production were 0.11 and 0.27 mg kg^{-1}, respectively, for measured values of freshly amended CL-20. The EC_{20} and EC_{50} values for freshly amended CL-20 were 0.05 and 0.15 mg kg^{-1}, respectively, for cocoons; and 0.04 and 0.12 mg kg^{-1}, respectively, for juveniles. Adult survival was not adversely affected in definitive studies with measured CL-20 soil concentrations up to and including 6.8 mg kg^{-1} in those studies utilizing freshly amended CL-20 (Table 4), and 5.0 mg kg^{-1} in the studies utilizing weathered and aged CL-20 in soil (Table 5). Furthermore, adult survival was not significantly (p>0.05) reduced in range-finding tests with nominal concentrations of freshly amended CL-20 up to and including 500 mg kg^{-1}. Therefore the results from these studies, focused on establishing benchmark values on the basis of sustaining earthworm populations (cocoon and juvenile production endpoints), did not additionally permit the establishment of an ecotoxicological benchmark value for survival of *E. fetida* adults exposed to CL-20 in soil.

Table 4. Ecotoxicological responses of the earthworm *Eisenia fetida* to CL-20 in freshly amended Sassafras sandy loam soil[a]

	CL-20 nominal concentrations (mg kg^{-1})											
Parameter	Control	0.01	0.02	0.03	0.06	0.08	0.1	0.25	0.5	0.75	1.0	5.0
CL-20 measured concentrations (initial mean (SE), mg kg^{-1} dry soil)	BDL	BDL	BDL	BDL	BDL	0.098 (0.027)	0.11 (0.003)	0.27 (0.001)	0.5 (0.03)	0.9 (0.03)	1.0 (0.01)	5.0 (0.09)
Adult survival/ replicate (%) (SE)	100	100	100	96 (0.3)	100	100	100	100	100	100	100	100
Adult final live mass/worm (g) (SE)	0.29 (0.01)	0.32 (0.01)	0.31 (0.01)	0.29 (0.01)	0.32 (0.01)	0.29 (0.01)	0.31 (0.01)	0.31 (0.01)	0.30 (0.01)	0.28 (0.01)	0.26[b] (0.01)	0.23[b] (0.01)
Cocoons mean total (SE)	17 (1.3)	22 (2.5)	21 (1.2)	22 (3.5)	15 (0.9)	15 (2.7)	12 [b] (0.5)	5 [b] (1.7)	2 [b] (1.3)	0.8 [b] (0.3)	0 [b]	0 [b]
Cocoons mean hatched (SE)	12 (1.6)	16 (2.5)	14 (1.3)	18 (2.2)	9 (1.2)	12 (2.6)	8 [b] (0.7)	2 [b] (0.7)	1 [b] (0.6)	0.5 [b] (0.3)	0 [b]	0 [b]
Juveniles mean (SE)	30 (4.6)	53 (4.7)	44 (5.8)	53 (6.9)	25 (4.4)	28 (6.0)	23 (2.2)	4 [b] (0.6)	2 [b] (1.9)	0.3 [b] (0.3)	0 [b]	0 [b]
No juveniles/hatched cocoons	2.6 (0.1)	3.5 (0.5)	3.2 (0.4)	2.9 (0.1)	2.7 (0.4)	2.7 (0.5)	3.0 (0.4)	2.3 (0.6)	1.8 (0.8)	1.0 (1.0)	0 [b]	0 [b]

[a] All soils were hydrated to 17.1 % dry mass soil 24 hr before start of test; n = 4 replicates; Control = acetone added as carrier solvent and evaporated before rehydration of soil; Adult final live mass/worm = least square mean adjusted for the covariate, initial live mass/worm, as determined by Analysis of Covariance (SYSTAT® 11.0); SE = standard error; BDL = below limit of detection (0.098 mg kg^{-1}).

[b] Significantly less ($p \leq 0.05$) than solvent controls within respective rows according to ANOVA and LSD means comparison (SYSTAT® 11.0).

Table 5. Ecotoxicological responses of the earthworm *Eisenia fetida* to CL-20 weathered and aged in Sassafras sandy loam soil[a]

	CL-20 nominal concentrations (mg kg^{-1})											
Parameter	Control	0.01	0.03	0.06	0.08	0.1	0.25	0.5	0.75	1.0	5.0	10.0
CL-20 measured concentrations, initial mean (mg kg^{-1} dry soil) (SE)	BDL	BDL	BDL	BDL	BDL	0.06 (0.002)	0.12 (0.026)	0.28 (0.008)	0.48 (0.013)	0.61 (0.018)	3.1 (0.01)	6.8 (0.014)
Adult survival/ replicate % (SE)	100	100	100	96 (0.03)	90 (0.03)	100	100	96 (0.03)	100	96 (0.03)	100	96 (0.03)
Adult final live mass/worm (g) (SE)	0.33 (0.01)	0.33 (0.01)	0.35 (0.01)	0.33 (0.01)	0.33 (0.01)	0.33 (0.01)	0.31 (0.01)	0.29 (0.01)	0.30 (0.01)	0.31 (0.01)	0.25 [b] (0.01)	0.23 [b] (0.01)
Cocoons mean total (SE)	22.0 (2.0)	16 (3.5)	11.0 [b] (1.9)	14.7 [b] (2.0)	11.5 [b] (2.6)	11.8 [b] (2.2)	13.0 [b] (1.5)	5.5 [b] (1.0)	1.0 [b] (1.0)	1.0 [b] (0.7)	0 [b]	0 [b]
Cocoons mean hatched (SE)	14.8 (1.4)	9.8 (2.7)	7.5 [b] (1.9)	7.7 [b] (1.8)	8.0 [b] (1.6)	9.0 [b] (1.7)	8.5 [b] (2.3)	3.8 [b] (0.6)	0.5 [b] (0.5)	0.3 [b] (0.3)	0 [b]	0 [b]
Juveniles mean (SE)	46.4 (10.9)	51.3 (4.5)	41.7 (8.5)	38.5 (2.9)	26.8 [b] (6.3)	29.8 [b] (8.4)	24.5 [b] (7.1)	7.5 [b] (1.8)	0.3 [b] (0.3)	0.3 [b] (0.3)	0 [b]	0 [b]
No juveniles/hatched cocoons	4.4 (0.4)	3.7 (0.4)	5.2 (1.5)	5.1 (1.3)	3.5 (0.6)	3.2 (0.4)	2.7 (0.2)	2.0 (0.3)	0.1 [b] (0.1)	0.3 [b] (0.3)	0 [b]	0 [b]

[a] All soils were weathered and aged by hydrating to 10.8% dry mass soil and allowing to dry in a greenhouse each week for 20 weeks, then hydrated to 17.1 % dry mass soil 24 hr before start of test; n = 4 replicates; Control = acetone added as carrier solvent and evaporated before rehydration of soil; Adult final live mass/worm = least square mean adjusted for the covariate, initial live mass/worm, as determined by Analysis of Covariance (SYSTAT® 11.0); SE = standard error; BDL = below limit of detection (0.06 mg kg^{-1}).

[b] Significantly less ($p \leq 0.05$) than solvent controls within respective rows according to ANOVA and LSD means comparison (SYSTAT® 11.0).

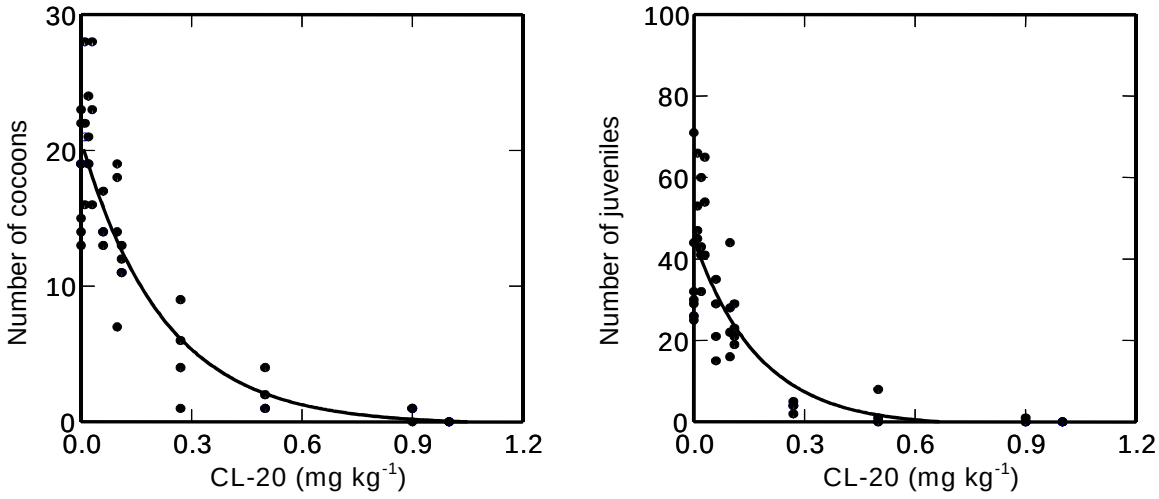

Figure 2. Effects of CL-20 on cocoon and juvenile production by *Eisenia fetida* in freshly amended Sassafras sandy loam soil

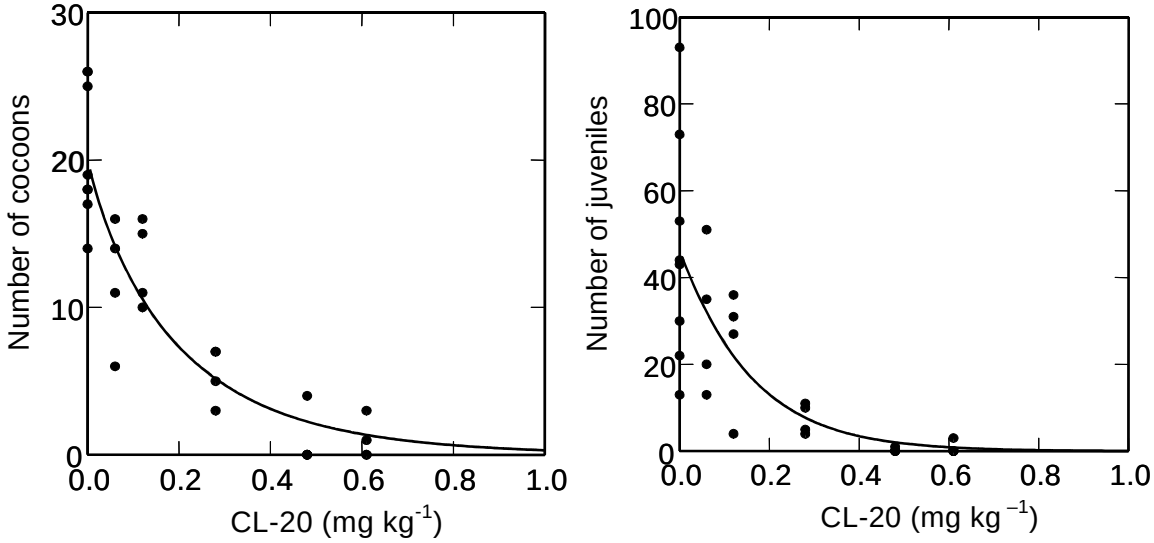

Figure 3. Effects of CL-20 weathered and aged in Sassafras sandy loam soil on cocoon and juvenile production by *Eisenia fetida*.

Table 6. Summary of toxicological benchmarks (mg kg^{-1} dry soil) determined for CL-20 freshly amended, and for CL-20 weathered and aged for 20 weeks, in Sassafras sandy loam soil using Earthworm Toxicity Test with *Eisenia fetida*.

CL-20 Exposure type	Adult survival				Cocoon production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended	5.0	>5.0	>5.0	>5.0	0.098	0.11	0.05	0.16	0.11	0.27	0.04	0.12
p or 95% CI	1.0				1.0	0.0002	0.03-0.07	0.09-0.27	0.593	0.001	0.02-0.06	0.05-0.19
Model (r^2)							Exponential (0.946)				Exponential (0.882)	
Weathered and aged	6.8	>6.8	≥6.8	≥6.8	0.01	0.03	0.036	0.13	0.06*	0.08*	0.038	0.112
p or 95% CI	1.0				0.645	0.001	0.005-0.067	0.075-0.184	0.374	0.031	0-0.094	0.029-0.195
Model (r^2)							Gompertz (0.936)				Gompertz (0.782)	

Table notes:

* These values are based on ecotoxicological responses in combination with nominal concentrations of CL-20 in soil. The remaining NOEC and LOEC values are based on ecotoxicological responses in combination with acetonitrile extraction of CL-20 and measurement by HPLC using USEPA Method 8330A. The ECp values are based on ecotoxicological responses in combination with nominal CL-20 concentrations for treatments below the limit of detection (BDL) and analytically determined values for treatments above the limit of detection based on acetonitrile extraction and measurement by HPLC using USEPA Method 8330A. Limit of detection was 0.098 mg kg^{-1} dry soil in freshly amended treatments, and 0.06 mg kg^{-1} in weathered and aged treatments. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) were derived from Analysis of Variance (ANOVA) and Fisher's Least Significant Difference (LSD) pairwise comparison tests using a significance level of $p \leq 0.05$. EC = effect concentration; CI = confidence intervals. ECp concentrations were extrapolated from nonlinear regression models that best fit each of the data sets using SYSTAT® 11.0. Exponential model: $Y = a \times e^{(([\log(1-p)] / ECp) \times C) + b}$. Logistic Gompertz model: $Y = a \times e^{([\log(1-p)] \times [C/ECp]b)}$. All analyses were performed using SYSTAT® 11.0 (SYSTAT Software, Inc., Point Richmond, CA).

Toxicity of CL-20 to the reproductive capacity of *E. fetida* in our studies is substantially greater than that previously established for RDX, HMX, or TNT. Table 7 shows that toxicity of CL-20 to juvenile production by *E. fetida* is at least one-to-two orders of magnitude greater than RDX or HMX in SSL soil under similar conditions (Simini et al. 2003). After weathering and aging, CL-20 remained highly toxic even in soils where CL-20 concentrations were below detectable levels, whereas the toxicities of RDX and HMX decreased in response to weathering and aging in soil (Table 7). The reason for this sustained toxicity of CL-20 in soil is intriguing, especially considering that only 44-to-67% of the original CL-20 remained (based on aggressive extraction with acetonitrile) after the weathering and aging process (Table 3). One possible explanation for sustained toxicity over time is that transformation products of CL-20 may form during the weathering and aging process that are at least as toxic as CL-20. The endproducts of CL-20 degradation in non-sterile or attenuated soils have commonly included nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitrogen gas (N_2), ammonia (NH_3), formate (HCOOH), and glyoxal (HCOCHO). Formation rates of the actual products depended on the specific abiotic or biotic processes (Balakrishnan et al. 2003; 2004a; Bhushan et al. 2003; 2004a, b; Trott et al. 2003; Hawari et al. 2004; Monteil-Rivera et al. 2004; Szecsody et al. 2004; Crocker et al. 2005). Several of these products, including glyoxal, ammonia, and formate, as well as reactive intermediates such as free radicals and amines (Balakrishnan et al. 2004b; Bhushan et al. 2004a, b; Hawari et al. 2004), can be formed during weathering and aging of CL-20 in SSL. These products can be more toxic to earthworms and other soil invertebrates compared with parent compound, thus potentially contributing to sustained toxicity observed in our study after weathering and aging CL-20 in soil. Resolving CL-20 degradation pathways that lead to formation of toxic products, their corresponding fates in aerobic soils, and assessment of the individual toxicities of degradation products to soil receptors require further investigations to better understand the mechanisms of CL-20 toxicity to earthworms in the soil vadose zone.

Table 7. Reproduction toxicity benchmarks (mg kg^{-1}) for explosive soil contaminants freshly amended or weathered and aged in Sassafras sandy loam soil determined using the same testing protocols for Earthworm Toxicity Test with *Eisenia fetida*.

Exposure/Benchmark	CL-20[a]	HMX[b]	RDX[b]	TNT[c]
Freshly amended				
EC_{50}	0.12	1.2	5.0	40
EC_{20}	0.04	0.4	1.6	33
Weathered and aged				
EC_{50}	0.11	>562[d]	14.9	9
EC_{20}	0.04	>532[d]	4.8	3

Table notes: [a] Data from this study; [b] Simini et al. 2003; [c] SERDP ER-1210; [d] The highest concentration tested.

Results of our studies showed that CL-20 reduced earthworm reproductive capacity, and growth of adult earthworms *Eisenia fetida*. Cocoon production, juvenile production, and adult growth were reduced by one-to-two orders of magnitude compared with the currently used nitramine explosives, RDX and HMX in Sassafras sandy loam soil. This soil type has low organic matter and clay contents thus supporting relatively high contaminant bioavailability, characteristics necessary for establishing conservative toxicity benchmarks that are protective of ecological receptors in soil when used to assess the potential toxicity of CL-20. Based on the results of the present studies, the order of toxicity of cyclic nitramines to *E. fetida* in SSL soil is (from greatest to least) CL-20 > HMX > RDX in freshly amended soil, and CL-20 > RDX > HMX for exposures after weathering and aging of these energetic compounds in soil. The order of toxicity of the three cyclic nitramines explosives in weathered and aged treatments parallels closely the order of their log K_{ow} values (1.92 CL-20 > 0.90 RDX > 0.17 HMX; Monteil-Rivera et al. 2004) suggesting that greater toxicity of CL-20 can be related, at least partially, to its greater hydrophobicity and affinity toward organic matter, which increases its potential to partition into soil biota, and greater bioavailability and uptake potentials compared with either RDX or HMX.

6.2.3 Effects of CL-20 on Potworm *Enchytraeus crypticus*

Test results complied with these validity criteria, defined in the ISO 16387 guideline (Section 5.7.2.2). Mean adult survival in negative controls was 100% in both definitive tests. The mean numbers of juveniles in negative controls of freshly amended, and weathered and aged treatments, respectively, were 1685 and 1973, and the coefficients of variation were 8 and 15 percent. Juvenile production in positive controls was reduced by 56 and 59% from respective negative controls, and was within ±2x the standard error of the positive control baseline established for the laboratory culture of *E. crypticus*. These results confirmed the power of the test, indicating that the toxicological effects determined in the definitive tests were most likely due to CL-20 treatments.

Definitive studies were conducted to assess the effects of CL-20 on the enchytraeid worm *E. crypticus*. Adult potworms were exposed in independent investigations to a range of CL-20 concentrations in freshly amended soil, and CL-20 weathered and aged in soil. Nominal concentrations of CL-20 in studies with freshly amended soils included 0, 0.08, 0.10, 0.25, 0.50, 0.75, 1.0, 5.0, and 10.0 mg kg^{-1}. Nominal concentrations of CL-20 weathered and aged in SSL soil included 0, 0.10, 0.25, 0.5, 0.75, 1.0, 5.0, and 10 mg kg^{-1}. Both survival and juvenile production by adult *E. crypticus* were affected in freshly amended SSL within the range of CL-20 concentrations tested (Table 8). For adult survival in freshly amended SSL, the bounded NOEC and LOEC values were 5 and 10 mg kg^{-1}, respectively. The EC_{20} and EC_{50} values for adult survival determined by logistic (Gompertz) model were 6 and 18 mg kg^{-1}, respectively. Juvenile production was the more sensitive measurement endpoint for assessing CL-20 toxicity to *E. crypticus* than adult survival. The bounded NOEC and LOEC values for juvenile production in freshly amended SSL were 0.11 and 0.27 mg kg^{-1}, respectively (Table 8). Concentration-response relationship for juvenile production determined by exponential model is shown in Figure 4. The EC_{20} and EC_{50} values for juvenile production were 0.1 and 0.3 mg kg^{-1}, respectively (Table 8).

Table 8. Summary of toxicological benchmarks (mg kg^{-1} dry soil) determined for CL-20 freshly amended, and for CL-20 weathered and aged for 20 weeks, in Sassafras sandy loam soil using Enchytraeid Toxicity Test with *Enchytraeus crypticus*.

CL-20 exposure type	Adult survival				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended	5	10	6	18	0.11	0.27	0.1	0.3
p or 95% CI	0.068	0.003	2-10	3-34	0.209	<0.0001	0.06-0.13	0.2-0.4
Weathered and aged	3.1	6.8	>6.8	>6.8	<0.06	0.06[b]	0.035	0.1
p or 95% CI	0.055	0.006	ND[a]	ND	ND	<0.0001	0.025-0.045	0.08-0.14

Table notes: Concentrations are based on acetonitrile extraction and HPLC using USEPA Method 8330A. Limit of detection was 0.098 mg kg^{-1} dry soil in freshly amended treatments, and 0.06 mg kg^{-1} in weathered and aged treatments. NOEC = no observed effect concentration; EC = effect concentration; CI = confidence intervals; [a] ND = parameter could not be determined within CL-20 concentration range tested; [b] Unbounded lowest observed effect concentration (LOEC) value.

The bounded NOEC and LOEC values for *E. crypticus* adults exposed to CL-20 weathered and aged in SSL were 3.1 and 6.8 mg kg^{-1}, respectively (Table 8). Weathering and aging of CL-20 in SSL increased the toxicity of test soil for *E. crypticus* adults by 147 percent based on the bounded LOEC values. Concentration-response relationship for adult survival could not be determined for this treatment because adult survival decreased by only 15 percent compared with carrier control at the highest CL-20 concentration tested. Similar to the results of exposure in freshly amended soil, juvenile production in soil containing CL-20 weathered and aged in situ was the more sensitive indicator of toxicity for *E. crypticus* compared with adult survival. This comports with results reported in literature for potworms (Schäfer and Achazi 1999; Dodard et al. 2003; Kuperman et al. 2003, 2004, 2005; 2006a,b,c; Römbke 2003). A statistically significant ($p < 0.0001$) reduction in number of juveniles, compared with carrier control, occurred in the lowest analytically verifiable treatment, producing an unbounded LOEC value of 0.06 mg kg^{-1} (Table 8). Increase in toxicity of weathered and aged CL-20 soil treatments was greater for the reproduction endpoint compared with adult survival, based on LOEC values (Table 8). The exponential model had the best fit for juvenile production data (Figure 4), generating EC_{20} and EC_{50} values of 0.035 and 0.1 mg kg^{-1}, respectively (Table 8). These values were significantly (95% CI) lower compared with CL-20 effects in freshly amended soil, indicating an approximately 300% increase in the toxicity of weathered and aged CL-20 treatments for reproduction of *E. crypticus*.

Toxicological benchmark data determined in this study for *E. crypticus* show that the toxicity of CL-20 was two orders of magnitude greater compared with TNT, and more then

five orders of magnitude greater compared with RDX (Table 9), based on EC_{50} values for reproduction in the similarly designed studies with SSL soil (Kuperman et al. 2003; 2005). The difference in toxicity to *E. crypticus* between CL-20 and HMX was even greater (Table 9), where *E. crypticus* was not affected by exposure to HMX up to the highest tested concentration of 21750 mg kg^{-1} HMX in SSL soil (Kuperman et al. 2003). Based on the results of the present studies, and those reported by others (Kuperman et al. 2003; 2005; 2006a), the order of toxicity of cyclic nitramines to *E. crypticus* in SSL soil is (from greatest to least) CL-20 > RDX > HMX. This order of toxicity of the three explosives parallels closely the order of their log K_{ow} values (1.92 CL-20 > 0.90 RDX > 0.17 HMX; Monteil-Rivera et al. 2004) suggesting that greater toxicity of CL-20 can be related, at least partially, to its greater hydrophobicity and affinity toward organic matter, which increases its potential to partition into soil biota, and greater bioavailability and uptake potentials compared with either RDX or HMX.

Table 9. Reproduction toxicity benchmarks (mg kg^{-1}) for explosive soil contaminants freshly amended or weathered and aged in Sassafras sandy loam soil determined using the same testing protocols for Enchytraeid Toxicity Test with *Enchytraeus crypticus*.

Exposure/Benchmark	CL-20[a]	HMX[b]	RDX[b]	TNT[c]
Freshly amended				
EC_{50}	0.3	>21,750[d]	51,413	98
EC_{20}	0.1	>21,750[d]	3,715	77
Weathered and aged				
EC_{50}	0.1	>17,498[d]	142,356	48
EC_{20}	0.035	>17,498[d]	8,797	38

Table notes: [a] Data from this study; [b] Kuperman et al. 2003; [c] Kuperman et al. 2005; [d] The highest concentration tested.

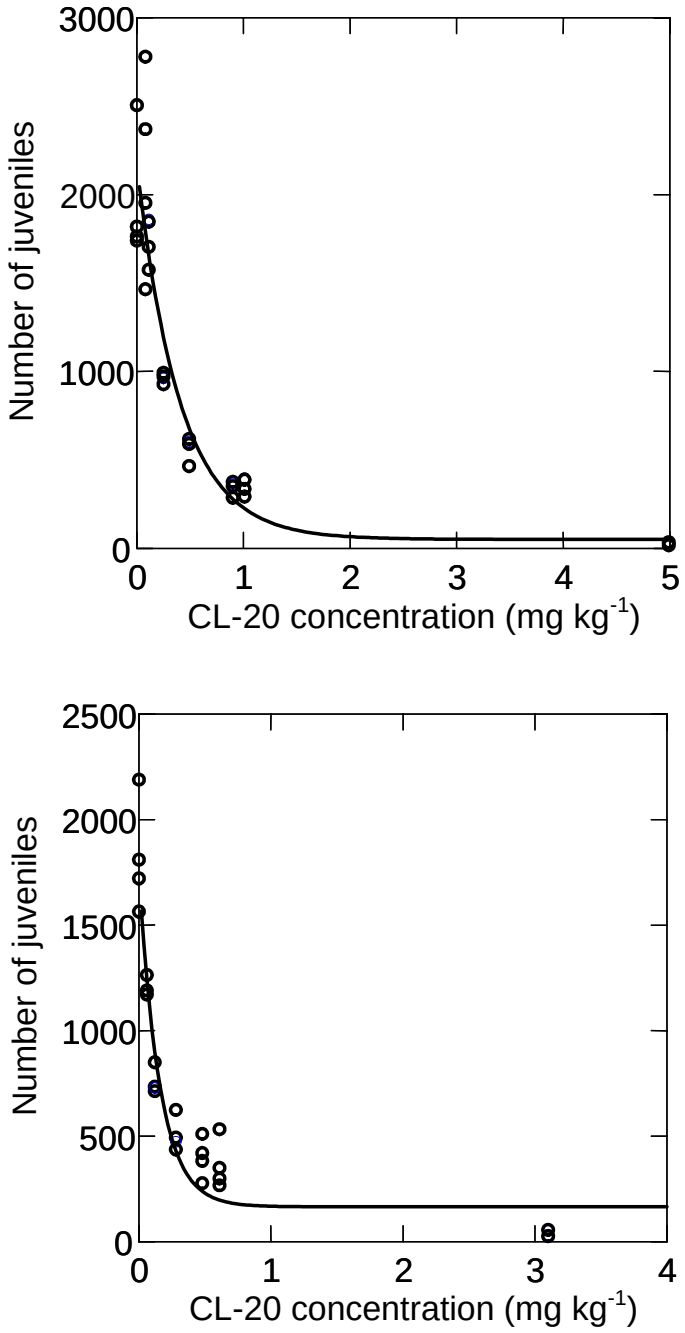

Figure 4. Effect of CL-20 on juvenile production by *Enchytraeus crypticus* in freshly amended Sassafras Sandy Loam (SSL) soil (top) and of CL-20 weathered and aged in SSL soil (bottom).

Notes (Figure 4): Concentration-response relationships were established using analytically determined CL-20 concentrations and exponential model $Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp}) \times C) + b$ ($r^2 \geq 0.948$). Weathering and aging of CL-20 included exposing hydrated amended soil in open glass containers in the greenhouse to alternating wetting and drying cycles for 20 weeks.

6.2.4 Effects of CL-20 on Collembolan *Folsomia candida*

Definitive toxicity assays using the Folsomia Reproduction Test were conducted to determine the effects of CL-20 on the reproduction of the Collembolan *F. candida*. Juvenile collembolans were exposed to a range of CL-20 concentrations in SSL soil in independent investigations. Nominal concentrations of CL-20, both in studies with freshly amended soils and of CL-20 weathered and aged in soil, included: 0, 0.25, 0.50, 0.75, 1, 5, 10, 25, 50, and 100 mg kg^{-1}. Measurement endpoints were assessed using multiple concentrations established from the results of range-finding tests, and included the number of surviving adults, and the number of juveniles produced, after 28 days of exposure. All ecotoxicological parameters were established using measured CL-20 concentrations for each treatment level.

Test results complied with the validity criteria adapted from the ISO 11267 test guideline (Section 5.7.2.3). Adult mortality in negative controls was ≤30% in all definitive tests, and averaged 10.0±0.3 (SE)%. Mean production of instars in negative controls exceeded 80 juveniles, averaging 217 instars in freshly amended soil and 112 instars in weathered and aged treatments. The coefficients of variation for the number of instars at the end of the definitive tests were ≤30%, with values averaging 14.7 and 12.3% in freshly amended, and weathered and aged treatments, respectively. Juvenile production in the positive controls (50 mg kg^{-1} Be) were decreased by 76 or 49% compared to negative controls in freshly amended or weathered and aged treatments, respectively. Results from both the negative controls and positive controls were within the baseline established for the laboratory culture of *F. candida*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to the CL-20 treatments. All reported ecotoxicological parameters have been calculated based on measured concentrations of CL-20 in soil. Measured concentrations of CL-20 are based on acetonitrile extraction using USEPA Method 8330A.

Both adult survival and juvenile production by *F. candida* were affected in freshly amended SSL within the CL-20 concentrations range tested (Table 10). For adult survival, the bounded NOEC and LOEC values were 0.9 and 1.0 mg kg^{-1}, respectively, in freshly amended soil; and 12.5 and 36 mg kg^{-1}, respectively, in weathered and aged CL-20 treatments (Table 10). The EC_{20} and EC_{50} values for adult survival determined by logistic (Gompertz) model were 0.7 and 32 mg kg^{-1}, respectively, in freshly amended soil; and 21 and 89 mg kg^{-1}, respectively, in weathered and aged CL-20 treatments (Table 10). Weathering and aging of CL-20 in SSL soil significantly (95% CI) decreased toxicity to adult *F. candida* based on EC_{20} values for survival, but this relationship was not statistically significant (95% CI) for the corresponding EC_{50} values for adult survival (Table 10).

Juvenile production was the more sensitive measurement endpoint for assessing CL-20 toxicity to *F. candida* compared with adult survival (based on the EC_{50} values and respective 95% CI from studies with freshly amended SSL soil, and on the EC_{20} values and respective 95% CI from studies with weathered and aged treatments), Table 10. The bounded NOEC and LOEC values for juvenile production were 0.5 and 0.9 mg kg^{-1}, respectively, in freshly amended SSL soil; and 12.5 and 36 mg kg^{-1}, respectively, in the weathered and aged treatment (Table 10). The concentration-response relationships for juvenile production that were determined by nonlinear regression analyses are shown in Figure 5. The exponential model had

the best fit for juvenile production data for freshly amended soil, and the logistic Gompertz model had the best fit for data from weathered and aged CL-20 treatments. The EC_{20} and EC_{50} values for juvenile production in freshly amended treatments were 0.2 and 0.7 mg kg^{-1}, respectively (Table 10). These values from weathered and aged treatments were 1 and 63 mg kg^{-1}, respectively. Despite the one-to-two orders of magnitude increase in EC_{20} or EC_{50} values for juvenile production in weathered and aged treatments compared with freshly amended treatments, the differences were not statistically significant (95% CI). This indicates that the 20-week weathering and aging process may not have been the major factor affecting the toxicity of CL-20 to *F. candida*, but it may have ecotoxicological implications (Fig. 5).

Table 10. Summary of toxicological benchmarks (mg kg^{-1} dry soil) determined for CL-20 freshly amended, and for CL-20 weathered and aged for 20 weeks, in Sassafras sandy loam soil using Folsomia Reproduction Test with *Folsomia candida*.

CL-20 Exposure type	Adult survival				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended	0.9	1.0	0.7	32	0.5	0.9	0.2	0.7
p or 95% CI	0.055	<0.0001	0-2	9-55	0.151	0.043	0.12-0.34	0.4-1.1
Weathered and aged	12.5	36	21	89	12.5	36	1	63
p or 95% CI	0.086	<0.0001	5-38	41-137	0.11	0.002	0-4	0-156

Table notes: Concentrations are based on acetonitrile extraction of CL-20 and determination by HPLC using USEPA Method 8330A. Limit of detection was 0.098 mg kg^{-1} dry soil in freshly amended treatments, and 0.06 mg kg^{-1} in weathered and aged treatments. NOEC = no observed effect concentration; EC = effect concentration; CI = confidence intervals.

Results of this investigation showed that in freshly amended soil the toxicity of CL-20 to *F. candida* was orders of magnitude greater than that of RDX, HMX, or TNT in the same soil type, based on EC_{50} and EC_{20} values for reproduction in similarly designed studies with SSL soil (Table 11). This was also true for comparisons of toxicity of CL-20 with toxicity of RDX or HMX in weathered and aged treatments. In contrast with toxicities of nitramine explosives RDX or HMX, the toxicities of CL-20 and the nitroaromatic explosive TNT were similar after weathering and aging in SSL soil based on the EC_{50} values (Table 11).

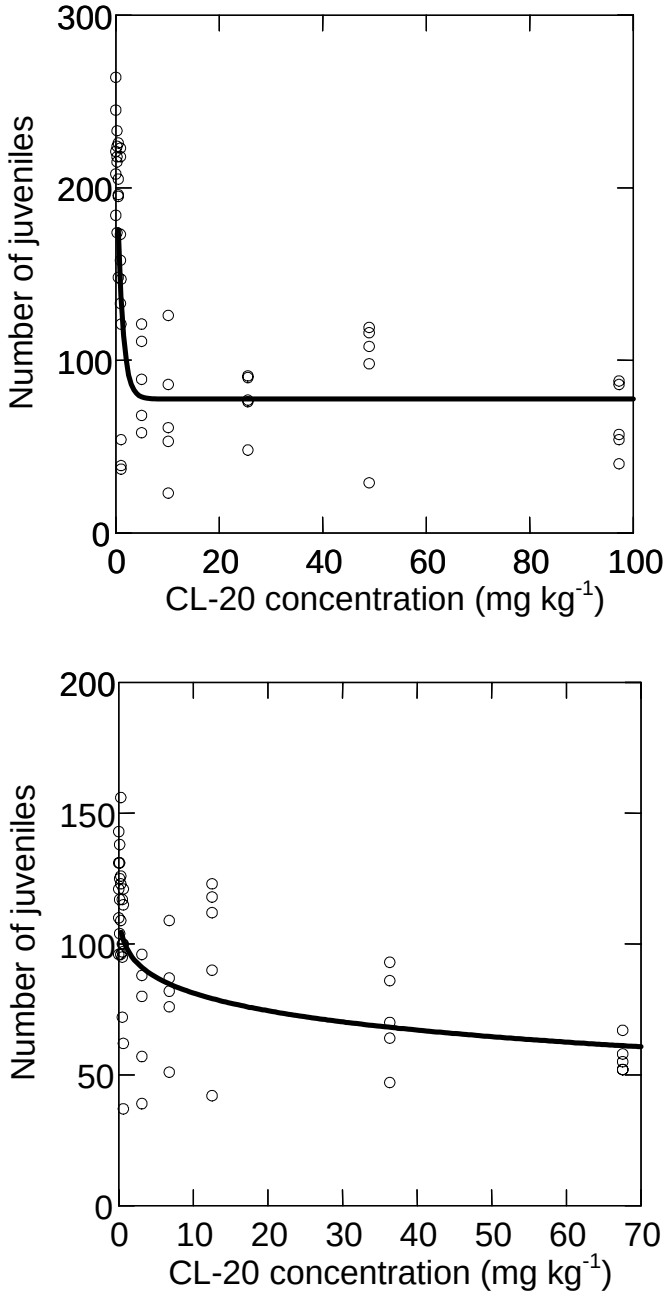

Figure 5. Effect of CL-20 on juvenile production by *Folsomia candida* in freshly amended Sassafras sandy loam (SSL) soil (top) and of CL-20 weathered and aged in SSL soil (bottom).

Notes (Figure 5): Concentration-response relationships were established using analytically determined CL-20 concentrations and exponential model (r^2=0.932) for freshly amended treatment, and Gompertz model (r^2=0.942) for weathered and aged treatment. Weathering and aging of CL-20 included exposing hydrated amended soil in open glass containers in the greenhouse to alternating wetting and drying cycles for 20 weeks.

Table 11. Reproduction toxicity benchmarks (mg kg^{-1}) for explosive soil contaminants freshly amended or weathered and aged in Sassafras sandy loam soil determined using the same testing protocols for Folsomia Reproduction Test with *Folsomia candida*.

Exposure/Benchmark	CL-20[a]	HMX[b]	RDX[b]	TNT[c]
Freshly amended				
EC_{50}	0.7	8,800	86	25
EC_{20}	0.2	235	28	17
Weathered and aged				
EC_{50}	63	10,400	770	61
EC_{20}	1	1,000	113	53

Table notes: [a] Data from this study; [b] SERDP CU-1221; [c] SERDP ER-1210.

Based on the results of the present studies, the order of toxicity of cyclic nitramines to *F. candida* in SSL soil is (from greatest to least) CL-20 > RDX > HMX. This order of toxicity of the three explosives parallels closely the order of their log K_{ow} values (1.92 CL-20 > 0.90 RDX > 0.17 HMX; Monteil-Rivera et al. 2004). This suggests that greater toxicity of CL-20 can be related, at least partially, to its greater hydrophobicity and affinity toward organic matter, which increases its potential to partition into soil biota, and greater bioavailability and uptake potentials compared with either RDX or HMX.

6.2.5 Discussion

Data for CL-20 toxicity to earthworm *E. fetida*, enchytraeid worm *E. crypticus*, and collembolan *F. candida* determined in the present study comport generally with data from the sparse number of available published studies of CL-20 effects on enchytraeids (Dodard et al. 2005), and the earthworm *Eisenia andrei* (Robidoux et al. 2004). Robidoux et al. (2004) found similar non-lethal effects on the earthworm *E. andrei* in response to CL-20 in SSL soil. The authors reported both LOEC and EC_{20} of 0.01 mg kg^{-1} for adult growth, cocoons, hatched cocoons, and juveniles. The EC_{50} values in their study were 0.04, 0.09, 0.07, and 0.05 mg kg^{-1} for adult growth, cocoons, hatched cocoons, and juveniles, respectively. Our study differed from the study by Robidoux et al. (2004) in that we did not observe statistically significant (p<0.05 or CI 95%) reductions in survival within the concentration ranges tested. Those authors reported LC_{20} and LC_{50} values of 25.3 and 53.4, respectively for adult *E. andrei*. Although survival was only reduced by 20% at nominal 500 mg kg^{-1}, the highest concentration in our range-finding study, the potential implications are that the population would not survive at concentrations ≥1 mg kg^{-1} in freshly amended soil due to elimination of reproductive capacity.

Dodard et al. (2005) reported lower benchmark values for *E. crypticus* in freshly amended SSL soil, including EC_{20} and EC_{50} values for juvenile production of 0.04 and 0.12 mg kg^{-1}, respectively based on nominal CL-20 concentrations. These values, in two soil formulations with considerably greater pH and organic matter content, ranged from 0.001 to 0.62 mg kg^{-1}

nominal CL-20 (Dodard et al. 2005). Reproduction toxicity benchmarks for *E. crypticus* determined in our study with freshly amended SSL soil were within the 95% CI range of those determined by Dodard et al. (2005) when he used a different enchytraeid species, *E. albidus*, in Rac50-50 soil formulation having 23% OM and pH 7.9, based on nominal CL-20 concentrations. Toxicity of CL-20 to adult potworm *E. crypticus* was greater compared with toxicity to the earthworm *E. andrei* based on NOEC, LOEC, LC_{20}, and LC_{50} values of 8.57, 90.7, 25.3, and 53.4 mg kg^{-1}, respectively, determined in a similar SSL soil freshly amended with CL-20 (Robidoux et al. 2004). Reproduction endpoints for earthworm *E. andrei* (cocoon production and viability, juvenile production) were more sensitive to CL-20 exposure in freshly amended SSL based on EC_{50} values that ranged from 0.05 to 0.09 mg kg^{-1} (Robidoux et al. 2004) compared with our results for *E. crypticus* or *F. candida*. However, these EC_{50} values were not statistically different (95% CI basis) from EC_{50} values for juvenile production by *E. crypticus* in weathered and aged CL-20 treatments determined in our study.

Soil type affected the toxicity of CL-20 to *E. crypticus* (Dodard et al. 2005) and to earthworm *E. andrei* (Robidoux et al. 2004). The reproduction toxicity benchmarks for *E. crypticus* were an order of magnitude lower (greater toxicity) in RacAg2002 soil formulation (41% OM, pH 8.2) compared with toxicity in SSL (Dodard et al. 2005). In contrast, all toxicological benchmarks for CL-20 were more than one order of magnitude greater (lower toxicity) for earthworm *E. andrei* in similarly formulated RacFor2002 soil, used in the study by Robidoux et al. (2004), compared with toxicity in SSL soil. The differential toxicity of CL-20 in these soil types suggests that future studies should include multiple soil types representing a broad range of soil properties (e.g., organic matter, clay content, pH) in order to assess the relationships among CL-20 toxicity, bioavailability, and soil properties.

Results of our studies showed that weathering and aging of CL-20 in SSL soil significantly increased the reproductive toxicity for exposed *E. crypticus* based on EC_{20} and EC_{50} values and respective 95% CI, and decreased mortality of adult *F. candida* based on EC_{20} values and respective 95% CI. Weathering and aging of CL-20 in SSL soil did not affect the toxicity for the earthworm *E. fetida* in our study compared with results of exposures in freshly amended soil. The effects of weathering and aging of contaminant explosives in soil on the exposure of terrestrial organisms, and resulting soil toxicities, have not been sufficiently investigated. No effect of weathering and aging of nitramines RDX or HMX in SSL soil on their toxicities were reported for *E. crypticus* (Kuperman et al. 2003) and *Eisenia fetida* (Simini et al. 2003). Kuperman et al. (2005; 2006b) reported that weathering and aging of nitroaromatic explosives and related compounds in SSL soil significantly ($p<0.05$ or CI 95%) increased the toxicity of TNT, or 2,6-dinitrotoluene (2,6-DNT), to *E. crypticus*, while toxicities of 2,4-dinitrotoluene (2,4-DNT), or 1,3,5-trinitrobenzene (TNB), were unaffected. In contrast, Dodard et al. (2003) found decreased TNT toxicity to *E. albidus* in the Organization for Economic Cooperation and Development (OECD) artificial soil following a 21-d aging period. Decreased toxicity to collembolan *F. candida* was reported for TNT aged in LUFA 2.2 soil at 60% of the WHC and 20°C in the dark (Schäfer 2002). Direct comparison of those results to findings of our studies is difficult due to several factors, including differences in molecular structures, toxicities, and fate in soil of these energetic contaminants compared to CL-20, other authors' use of different test species, different soil types, and shorter or undefined aging periods.

Specific mechanisms for changes in the toxicity following weathering and aging of CL-20 in soil are not well understood. Compounds produced due to CL-20 degradation or transformation during the weathering and aging process may be more toxic or more bioavailable to soil organisms compared with the parent material, and these may be factors contributing to the increased toxicity in weathered and aged treatments. Increased toxicity of metabolic byproducts of energetic soil contaminants was demonstrated by Lachance et al. (2004) in a study investigating the effects on adult earthworm *E. andrei* of TNT and its reduction products 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT). Those authors reported LC_{50} values for adult mortality from exposure to TNT, 4-ADNT, or 2-ADNT, of 132, 105, and 215 mg kg^{-1}, respectively, and gave the following order of toxicity: 4-ADNT > TNT > 2-ADNT. Our study does not allow determining whether increased toxicity to *E. crypticus* of weathered and aged CL-20 in soil was caused by formation of its degradation products, as was demonstrated for TNT by Lachance et al. (2004), or alteration of bioavailability of the parent chemical. Information on degradation pathways of CL-20, and particularly on its fate in a variety of representative soil types and components, is limited because many of the investigations of this relatively new compound are ongoing (Bhushan et al. 2003, 2004a, b, c; Trott et al. 2003; Balakrishnan et al. 2004a, b; Hawari et al. 2004; Qasim et al. 2004; Szecsody et al. 2004).

Szecsody et al. (2004) reported that CL-20 abiotically degrades in oxic environments in the presence of 2:1 phyllosilicate clays (hectorite, montmorillonite, nontronite), micas (biotite, illite), and specific oxides (MnO_2, and the ferrous-ferric iron oxide magnetite). CL-20 was also found to be photoreactive when exposed to sunlight (Hawari et al. 2004; Szecsody et al. 2004). A few studies have shown that CL-20 can be biodegraded in aerobic surface soils (Jenkins et al. 2003; Trott et al. 2003) by bacteria isolated from these soils (Bhushan et al. 2003; Trott et al. 2003), and by functionally diverse bacterial enzymes (Bhushan et al. 2004a, b). Crocker et al. (2005) has shown that CL-20 is susceptible to both biotic and abiotic degradation in aerobic soils, with relatively rapid rates of CL-20 biodegradation (half-lives = 0.6-to-31.5 d) in surface and subsurface soils. Similar rates of CL-20 biodegradation were observed with garden and agricultural soils (Trott et al. 2003), however considerably slower rates of CL-20 degradation (half-lives = 144-to-686 d) were observed by Jenkins et al. (2003) with soils from three firing ranges. The end products of CL-20 degradation in non-sterile or attenuated soils have commonly included nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitrogen gas (N_2), ammonia (NH_3), formate (HCOOH), and glyoxal (HCOCHO), although formation rates of the actual products depended on the specific abiotic or biotic processes (Balakrishnan et al. 2003, 2004b; Bhushan et al. 2003, 2004a, c; Trott et al. 2003; Hawari et al. 2004; Monteil-Rivera et al. 2004; Szecsody et al. 2004; Crocker et al. 2005). Several of these products, including glyoxal, ammonia, and formate, as well as the early reactive intermediates such as free radicals and imines (Balakrishnan et al. 2004a; Bhushan et al. 2004b,c; Hawari et al. 2004) can be formed during weathering and aging of CL-20 in SSL. These products of CL-20 degradation can be more toxic to enchytraeids compared with parent compound, thus potentially contributing to greater toxicity observed in our study with weathered and aged CL-20 treatments. Resolving CL-20 degradation pathways that lead to formation of toxic products, their fate in aerobic soils, and assessment of the individual toxicities of degradation products to soil receptors require further investigations to better understand the mechanisms of CL-20 toxicity in the soil vadose zone.

6.2.6 Effects of CL-20 on the Soil Microinvertebrate Community

Results of analytical determination showed that the initial concentrations of CL-20 averaged 96±3 percent of nominal concentrations thus confirming the precision of the treatment preparation procedures (Table 12). Concentrations of CL-20 in SSL soil treatments remained relatively stable during the 12-week microcosm study averaging 93±2, 96±4, and 97±4 percent on the initial concentration in freshly amended soil after 4, 8, and 12 weeks, respectively (Table 12).

Total abundance of microarthropods was not affected (Student's *t*-Test $p = 0.490$) by the soil preparation procedures based on comparison of the baseline data (1.1±0.1 ind g^{-1}, n=5) and data from control samples extracted after the first harvest (0.96±0.2 ind g^{-1}, n=4). The abundance of nematodes in similarly replicated samples was significantly (Student's *t*-Test $p = 0.025$) greater in control (34.3±4.5 ind g^{-1}) after the first harvest compared with baseline data (17.2±1.8 ind g^{-1}). Overall numbers of microarthropods and nematodes increased in control treatments by 242 and 423%, respectively by the end of the 12-week study. These results confirmed that soil preparation procedures and the controlled abiotic environmental conditions in test containers (soil moisture, temperature, RH, photoperiod, etc.) did not adversely affect the soil microinvertebrate community in SSL soil during assessment of the effects of exposure to CL-20.

Exposure to CL-20 had an immediate adverse effect on the microarthropod community (Figures 6, 7). Total numbers of microarthropods were significantly (p=0.007) decreased in the first concentration tested (105 mg kg^{-1}) and remained significantly (p<0.0001) lower compared with control in the remaining CL-20 treatments after 4 weeks of exposure. After 8 weeks, total numbers of microarthropods were significantly ($p \leq 0.033$) lower in and above the 5236 mg kg^{-1} treatment. By the end of 12-week exposure to CL-20, the total abundance of microarthropods was not significantly ($p \geq 0.123$) different from control in any of CL-20 treatments, although total numbers continued to decline in a concentration-dependent manner.

The selected concentration range of CL-20 adequately assessed the concentration-response relationships for total microarthropods in SSL soils (Figure 7). Similar relationships were established for individual microarthropod groups (graphics are not shown) and allowed us to determine group-specific ecotoxicological benchmarks for CL-20. High variability in the abundance of microarthropods, which is common for natural soil ecosystems, produced relatively wide ranges of the 95% CI for endpoint estimates for all taxa except mesostigmatid mites (Table 13). This predatory group of mites had the lowest estimated EC_{50} values on the first and third harvests, but was preceded by oribatid mites on the second harvest. The final order of EC_{50} values (from lowest to greatest) after 12 weeks was Mesostigmata > Collembola > Prostigmata > Oribatida (Table 13).

Table 12. Nominal and analytically determined CL-20 concentrations in Sassafras sandy loam used in the 12-week microcosm study with indigenous soil microinvertebrate community.

Nominal mg kg^{-1}	Initial determined mg kg^{-1}		Harvest I mg kg^{-1}		Harvest II mg kg^{-1}		Harvest III mg kg^{-1}	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
100	108	4	105	6	90	5	91	4
500	479	18	480	19	385	32	485	13
1000	870	14	836	36	843	33	1021	23
2500	2355	61	2211	132	2318	141	2392	136
5000	4635	235	4068	709	5236	472	4251	227
7500	7020	268	6345	218	7032	218	6214	442
10000	10300	303	8689	727	10011	291	9615	844

Table notes:
Data are means and standard errors (S.E.); n=3. Replicate microcosm units were harvested at four-week intervals. Concentrations are based on acetonitrile extraction of CL-20 and determination by HPLC using USEPA Method 8330A. BDL = below detection limit. Method detection limit was 0.06 mg kg^{-1}.

The overall composition of microarthropod community in SSL soil was not affected by exposure to CL-20 based on the number of taxonomic group present in the individual treatments after 12 weeks (Figure 6). Community structure analysis revealed greater sensitivity to CL-20 by predatory mesostigmatid mites, which were absent in 6345 mg kg^{-1} treatment after 4 weeks, and in the 843, 2318, 7032, and 10011 mg kg^{-1} treatments after 8 weeks of exposure to CL-20 (Figure 8). By the end of the 12-week exposure, mesostigmatid mites were present in all CL-20 treatments (Figures 6, 8) although their relative abundances (expressed as percent of total abundance) remained lower compared with control (Figure 8). The order of relative abundance of microarthropods (from greatest to least) was Prostigmata > Oribatida ≥ Collembola > Mesostigmata (Table 13, Figure 6). Soil arthropods belonging to classes Diplopoda and Symphyla, and the insect orders Diplura, Psocoptera, Thysanóptera, Coleoptera, and Diptera were found among most CL-20 treatments after 4 and 8 weeks of exposure but disappeared from all treatments (including control) after 12 weeks.

Indigenous microarthropod and nematode communities showed contrasting sensitivities to CL-20 in SSL soil. Total numbers of nematodes were either unaffected (p>0.05) or significantly (p<0.5) increased in CL-20 treatments compared with control. Relative abundances of trophic groups of nematodes varied among harvest dates (Figure 9). After the 8-week exposure, bacterivore and fungivore nematodes equally dominated (44±2%) the nematode community followed by omnivore (4.6±0.5%), hatchling (3.3±0.3%), herbivore (1.6±0.3%), and predator (1.6±0.8%) groups. Relative abundance of bacterivore nematodes increased over time to 66±3 and 74±2% after 8 and 12 weeks, respectively. Similarly, relative abundance of hatchling group increased over time to 3.7±0.6 and 5.2±0.8% after 8 and 12 weeks. Correspondingly, respective relative abundances of fungivore, omnivore, herbivore, and predator groups decreased after 8 and 12 weeks to 26±2.5 and 20±2.7%, 2.4±0.3 and 0.7±0.2%, 0.3±0.2 and 0.2±0.03%, and 0.6±0.2 and 0.3±0.2%, respectively. Correspondently, respective relative abundances of

fungivore, omnivore, herbivore, and predator groups decreased to 26±2.5 and 20±2.7%, 2.4±0.3 and 0.7±0.2%, 0.3±0.2 and 0.2±0.03%, and 0.6±0.2 and 0.3±0.2% after 8 and 12 weeks, respectively.

Only predator group among nematodes was consistently adversely affected by exposure to CL-20 (Figure 8). The abundance of predatory nematodes decreased in a concentration-dependent manner throughout the 12-week exposure producing the EC_{50} values of 31, 55, and 68 mg kg^{-1} after 4, 8 and 12 weeks, respectively (Table 13). Similar greater sensitivity of predatory nematodes to chemical exposures, compared with other trophic groups of the nematode community, was observed in a study with copper and *p*-Nitrophenol by Parmelee et al. (1993). Intermittent significant ($p<0.5$) decreases compared with control treatment were found for omnivore nematodes in the 2211 mg kg^{-1} treatment after 4 weeks, and in the 485 and 2392 mg kg^{-1} treatments after 12 weeks. The abundance of herbivore nematodes after 8 weeks was significantly ($p=0.01$) lower at-and-above the 843 mg kg^{-1} treatment-level, compared with control. Detection of these group-specific responses demonstrated the advantage of trophic structure analysis compared with relying only on total abundance numbers in identifying the effects of CL-20 on the soil microinvertebrate community.

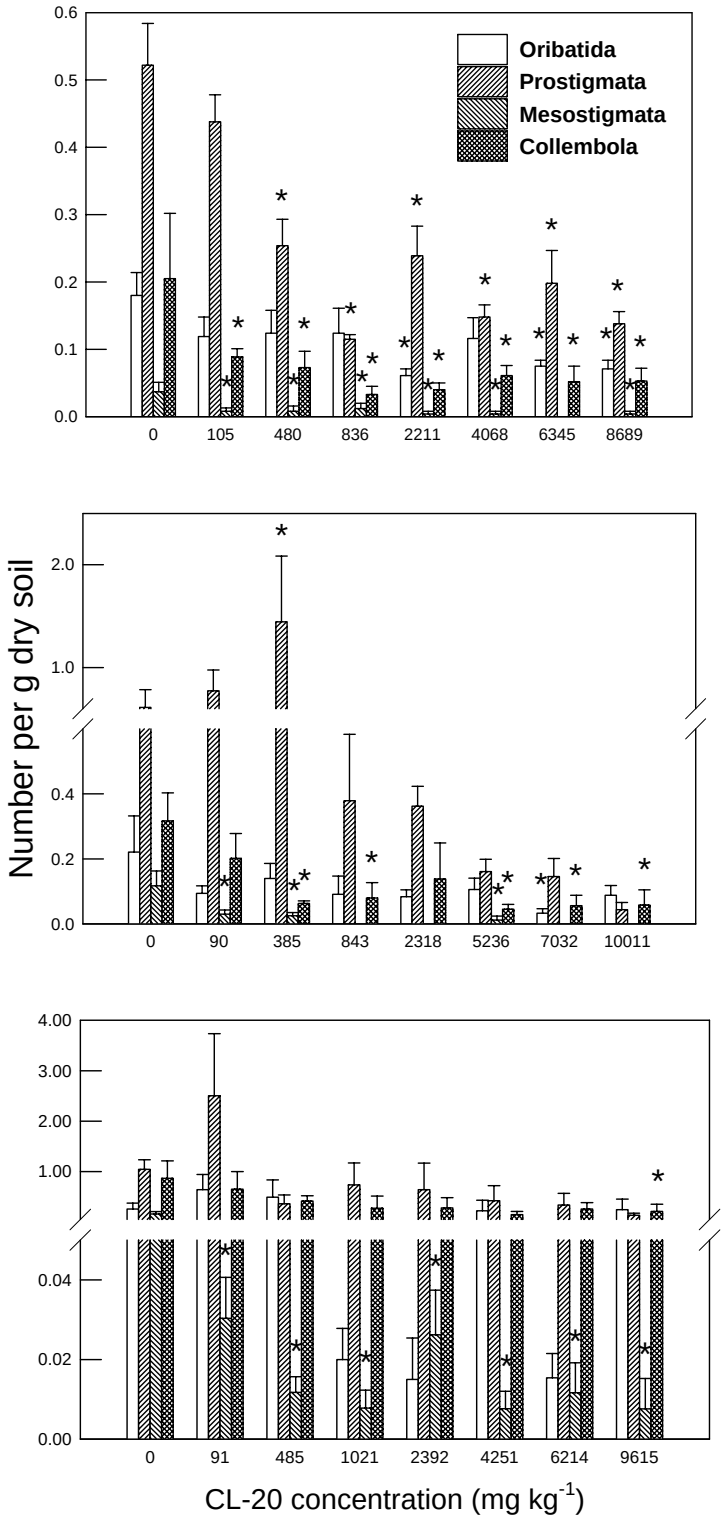

Figure 6. Abundance of microarthropod groups after 4 (top), 8 (middle), and 12 weeks (bottom) of exposure to CL-20 in Sassafras sandy loam soil. Significant ($p \leq 0.05$, Fisher's LSD) change from control is indicated by [*].

Figure 7. Effect of CL-20 on total abundance of microarthropods after 4 (top), 8 (middle), and 12 (bottom) weeks of exposure to CL-20 in Sassafras sandy loam soil. Concentration-response relationships were established using analytically determined CL-20 concentrations and exponential model $Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp}) \times C) + \mathrm{b}$.

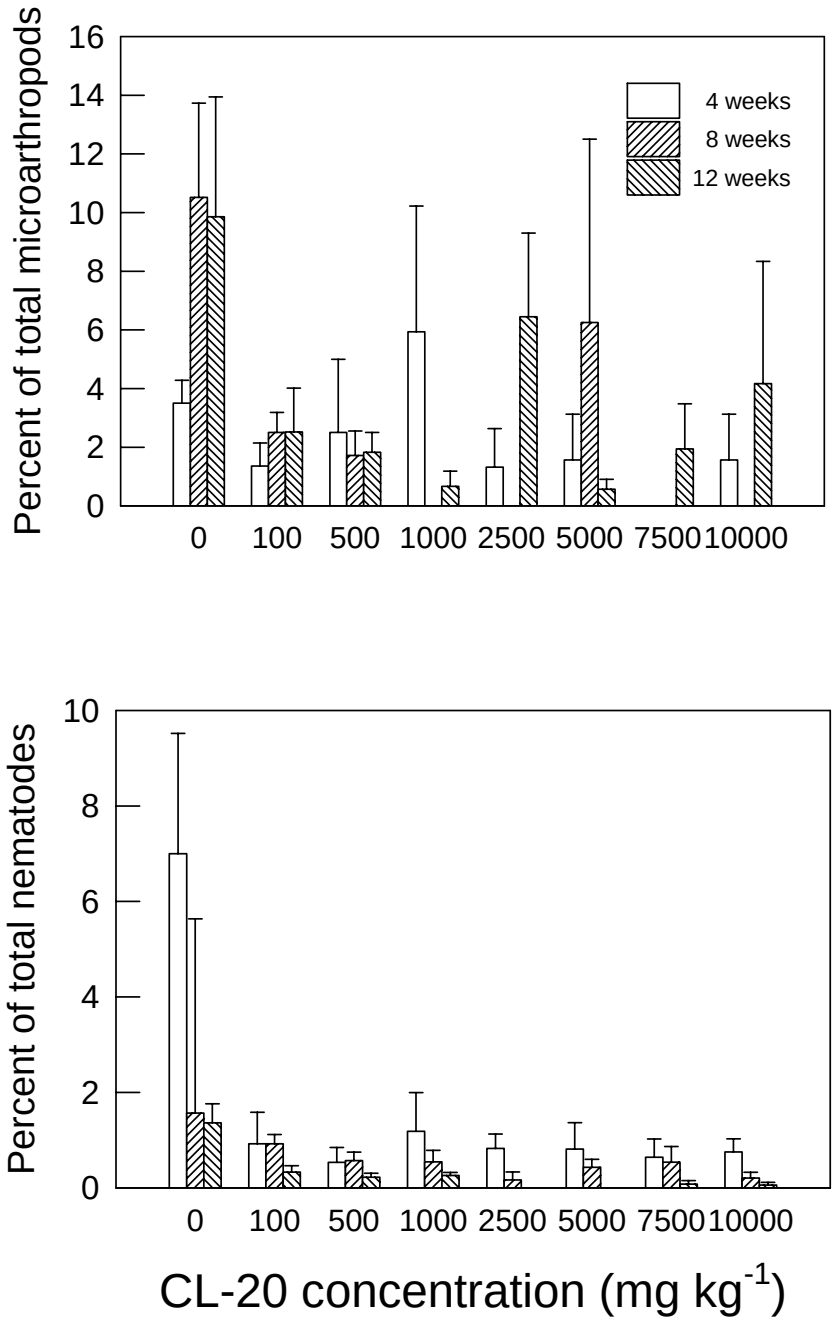

Figure 8. Relative abundance of predatory mesostigmatid mites (top) and predatory nematodes (bottom) in Sassafras sandy loam soil amended with CL-20. Values are means and standard errors (n=4). Nominal CL-20 concentrations are shown. Analytically determined concentrations for each exposure period are reported in Table 3.

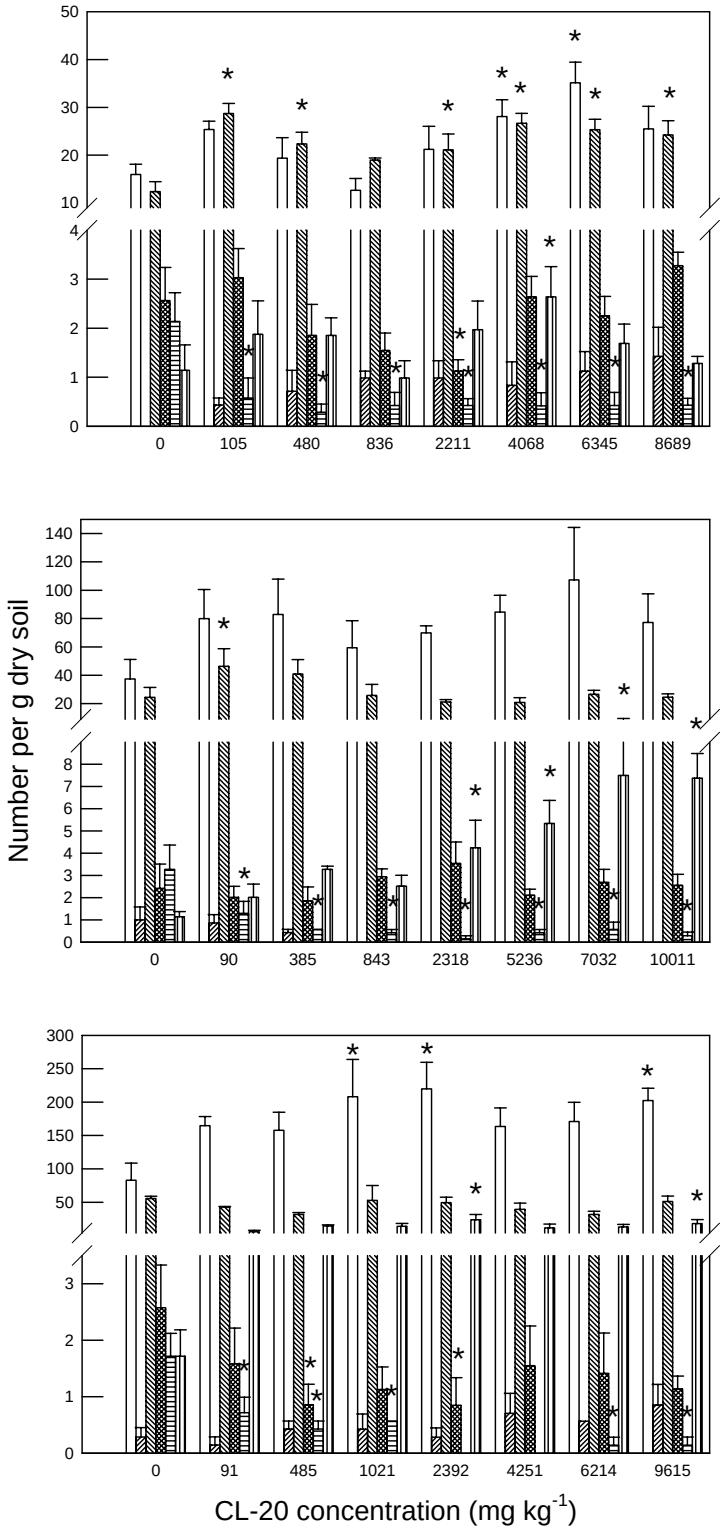

Figure 9. Abundance of trophic groups of nematodes after 4 (top), 8 (middle), and 12 (bottom) weeks of exposure to CL-20 in Sassafras sandy loam soil. Significant (p<0.05, Fisher's LSD) change from control is indicated by [*].
Legend: Bacterivore , Herbivore , Fungivore , Omnivore , Predator , Hatchling .

Table 13. Toxicity benchmarks and relative abundance of soil microinvertebrates established in a microcosm study after the 4, 8, and 12 weeks of exposure in CL-20 amended Sassafras sandy loam soil.

Invertebrate group	4 weeks				8 weeks				12 weeks			
	EC_{20} mg/kg	EC_{50} mg/kg	r^2	Percent of total*	EC_{20} mg/kg	EC_{50} mg/kg	r^2	Percent of total	EC_{20} mg/kg	EC_{50} mg/kg	r^2	Percent of total
Oribatida	166	516	0.999	26(3)	6	19	0.998	20(5)	185	575	0.984	16(6)
95% CI	0-466	0-1446			0-48	0-149			0-601	0-1867		
Prostigmata	63	197	0.999	55(3)	691	2145	0.979	59(7)	88	274	0.942	47(7)
95% CI	20-107	62-332			0-2182	0-6777			0-319	0-991		
Mesostigmata	10	31	0.999	2.2(0.6)	14	42	0.999	2.6(1.4)	10	31	0.999	3.5(1.1)
95% CI	0-30	0-95			0.2-27	0.6-84			0.4-20	1.3-61		
Collembola	18	55	0.999	16(1)	29	90	0.998	19(3)	47	146	0.975	28(4)
95% CI	0-43	0-132			0-72	0-225			0-218	0-677		
Total microarthropods	54	168	0.998	100	428	1329	0.980	100	96	300	0.932	100
95% CI	16-92	50-285			0-1068	0-3317			0-299	0-928		
Predatory nematodes	10	31	0.977	1.6(0.8)	18	55	0.719	0.6(0.2)	22	68	0.985	0.3(0.2)
95% CI	0-27	0-85			2-34	5-104			2-42	7-130		

Table notes:

* Numbers representing relative abundances of individual groups are mean percent (and standard errors, n=8) of the total (not reported in this table) microarthropod or nematode numbers in all treatments for each exposure period. Concentration-response relationships were established using analytically determined CL-20 concentrations and exponential model $Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp}) \times C) + b$. EC = effect concentration; CI = confidence intervals. Concentrations are based on acetonitrile extraction and HPLC using USEPA Method 8330A. Method detection limit was 0.06 mg kg^{-1}.

The contrasting effects of exposure to CL-20 by soil microarthropods and nematodes can be related to differences in chemical environments of soil microsites inhabited by each of these organisms (e.g., the water film around soil particles, and air-filled soil pores). These differences demonstrated that chemical bioavailability can affect the exposure and resulting toxicity of CL-20 to the two distinct groups of the soil invertebrate community. Exposure of nematodes to sparingly water-soluble CL-20 (3.7 mg L^{-1} at 25°C; Monteil-Rivera et al. 2004) could be decreased because they occupy soil pore water and water films surrounding soil particles. This decreased exposure for nematodes has likely contributed to the significantly lower toxicity of CL-20 to nematodes compared to microarthropods, which occupy soil surfaces in air-filled soil pores and thereby received greater exposure to CL-20 in soil.

Special consideration in assessing CL-20 toxicity to the soil invertebrate community was given to the effects of weathering and aging of CL-20 in soil on the exposure of soil receptors. The duration of microcosm assay and chronosequential harvesting of subset replicate units at 4-week intervals allowed the assessment of the CL-20 toxicity to include the acute effects of freshly amended CL-20 that approximated exposure conditions in standardized single-species tests, and the chronic effects of prolonged exposure that integrated the weathering and aging of CL-20 in SSL soil, and more closely approximated the exposure effects in the field locations, where CL-20 may persist for extended periods of time. The changing exposure conditions during the 12-week study included potential alteration of CL-20 bioavailability in soil for microinvertebrates. This was evident from the toxicity data for select groups of microarthropods and nematodes. The EC_{50} values for Collembola steadily increased (decreasing toxicity) from 55 to 90, and 146 mg kg^{-1} after 4, 8, and 12 weeks of exposure, respectively. The corresponding values for prostigmatid mites increased from 197 to 2145 and 274 mg kg^{-1} after 4, 8, and 12 weeks of exposure, respectively. Similar increases in EC_{50} values (from 31 to 68 mg kg^{-1}) were established for predatory nematodes. In contrast, the toxicity of CL-20 to oribatid mites increased after 8 weeks (lower EC_{50} value) compared with toxicity after 4 weeks, but later returned to approximately their initial level (Table 13). These results show the complexity of possible interactions among the fate processes of weathering and aging of CL-20 in soil, changes in bioavailability of parent material and its possible degradation products (Balakrishnan et al. 2003, 2004a, b; Trott et al. 2003), and the effects on different taxonomic groups that represent a range of sensitivities, which often correlate with physiologically determined mechanisms of toxicity varying among taxa. The inclusion of the weathering and aging component in the CL-20 toxicity assessments allowed us to incorporate potential alterations in the soil chemical environment, and corresponding changes in toxicity at contaminated sites, into the development of toxicological benchmarks for the soil invertebrate community.

Overall toxicity data for soil microarthropods and predatory nematodes established in this microcosm assay were comparable with mortality data established in our standardized single-species toxicity tests with soil invertebrates earthworm *Eisenia fetida* (ISO 11268-2:1998), potworm *Enchytraeus crypticus* (ISO 16387:2001), and collembolan *Folsomia candida* (ISO 11267:1998) exposed to CL-20 in a similar SSL soil, and summarized in Table 14. Results of the present study also comport generally with mortality data from the available published studies of CL-20 effects on the earthworm *Eisenia andrei* (Robidoux et al. 2004) and enchytraeids (Dodard et al. 2005, Kuperman et al. 2006a). Robidoux et al. (2004) reported LC_{50} value of 53.4 mg kg^{-1} (46-63, 95% CI) for adult *E. andrei* in a similar SSL soil, and an

unbounded NOEC value of 125 mg kg^{-1} in a forest soil formulation RacFor2002 (41% OM, pH 8.2) following the 4-week exposure to CL-20 in freshly amended soils. Lower LC_{50} values ranging from 0.1 to 0.7 mg kg^{-1} for survival of adult *E. crypticus*, and LC_{50} values ranging from 0.2 to >1.0 mg kg^{-1} for survival of adult *E. albidus*, were established by Dodard et al. (2005) in freshly amended SSL soil or in formulated soils with greater OM contents. However, reproduction endpoints for *E. andrei* (cocoon production and viability, juvenile production) were more sensitive to CL-20 exposure in freshly amended SSL, based on EC_{50} values that ranged from 0.05 to 0.09 mg kg^{-1} (Robidoux et al. 2004) compared with our results for the soil invertebrate community. Similarly, reproduction toxicity benchmarks (EC_{50}) for enchytraeids and collembola ranging from 0.08 to 0.7 mg kg^{-1} (Dodard et al. 2005; Kuperman et al. 2006a, this report) indicated greater toxicity of CL-20 compared with data from our microcosm assay. This suggests that a longer-term exposure of the microinvertebrate community to CL-20 in a microcosm designed to include assessment of the effects on reproduction can reveal greater toxicity than that ascertained in our study.

Table 14. Toxicity benchmarks for soil invertebrates established in standardized single-species toxicity tests with CL-20 amended Sassafras sandy loam soil.

Species		LC_{50}	EC_{50}
Eisenia fetida		>500	0.1
	95% CI	ND	0.07-0.13
Enchytraeus crypticus		18	0.3
	95% CI	2.6-34	0.2-0.4
Folsomia candida		32	0.7
	95% CI	9-55	0.36-1.06

Table notes:
Toxicity benchmarks for mortality (LC_{50}) and reproduction (EC_{50}) endpoints were determined on the basis of concentration-response relationships in our previous studies with earthworm *Eisenia fetida* (ISO 11268-2:1998), potworm *Enchytraeus crypticus* (ISO 16387:2001), and collembolan *Folsomia candida* (ISO 11267:1998) using similar Sassafras sandy loam soil freshly amended with CL-20. ND = not determined; no concentration-response relationships for mortality within tested concentration range.

6.2.7 Effects of CL-20 on Litter Decomposition

Litter decomposition is one of the most integrating processes within the soil ecosystem because it involves complex interactions of soil microbial and faunal activity with the soil chemical environment. Populations of species from different taxonomic groups contribute to the breakdown of plant residues. Any disturbance that alters litter decomposition can result in

nutrient losses and a decline in soil fertility. Therefore, an assessment of how an accidental release of CL-20 in soil may alter rates of litter decomposition, and the subsequent rates of nutrient retention and release, is critical to understanding its potential impacts on the overall functioning of the soil ecosystem.

Concentrations of CL-20 in soil treatments remained relatively stable during the 8-month study, and ranged on average from 88 to 102 percent of the initial concentrations in freshly amended soil (Table 15). Results showed that, except for a short-term decrease (p=0.001) in the nominal 100 mg kg^{-1} CL-20 treatment after 4 months, there was no decrease in litter decomposition rate in any of the CL-20 treatments compared with control (Figure 10). Litter decomposition was actually significantly (p<0.05) stimulated in several CL-20 treatments (nominal mg kg^{-1}) including 7500 after 1 month, 1000 after 2 months, and 2500 after 4 and 5 months. By the end of the 8-month study, mass loss by Orchard grass litter was significantly (p<0.05) greater in the nominal 100, 1000, and 7500 CL-20 soil treatments compared with control.

Litter decay rate constants, which integrate mass loss data over the entire study period, were statistically similar (p>0.05) in all CL-20 treatments except in the nominal 2,500 mg kg^{-1} CL-20 in which k value was significantly (p=0.029) greater compared with control or other CL-20 treatments tested in this study (Table 16). The increase in decay rate in the 2,500 mg kg^{-1} CL-20 treatment was due to a greater litter mass loss on the 4th and 6th month harvest dates (Figure 10).

Table 15. Initial analytically determined concentrations (means, n=3) and percent recovery of CL-20 at each of the six harvests of *Dactylus glomerata* litter after 1, 2, 3, 4, 6, and 8 months of incubation in Sassafras sandy loam soil.

Initial mg kg^{-1}	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 5	Harvest 6
BDL	BDL	BDL	BDL	BDL	BDL	BDL
108	97	84	85	88	84	70
479	100	81	101	113	104	106
870	96	97	117	97	97	95
2355	94	98	102	94	86	84
4635	88	103	96	105	92	97
7020	89	100	82	111	82	86
10300	84	97	93	105	85	80
Mean	**93**	**94**	**97**	**102**	**90**	**88**
S.E.	**2.2**	**3.2**	**4.4**	**3.5**	**3.0**	**4.5**

Table notes: BDL = below detection limit. Method detection limit was 0.06 mg kg^{-1}.

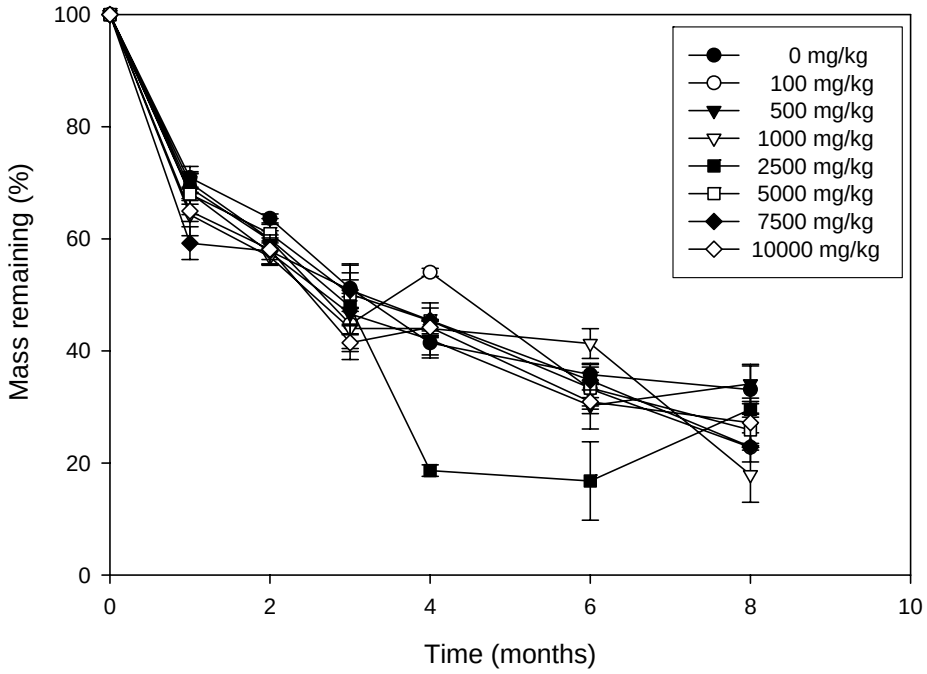

Figure 10. Decomposition of Orchard grass (*Dactylus glomerata)* litter in CL-20 amended soil.

Table 16. Litter decomposition parameters for *Dactylus glomerata* established in the 8-month microcosm study with CL-20 amended Sassafras sandy loam soil.

CL-20 mg kg^{-1}	*k*	S.E.	r^2	*p*
0	-1.616	0.236	0.904	1.000
100	-2.003	0.231	0.938	0.262
500	-1.620	0.312	0.843	0.947
1000	-2.108	0.340	0.885	0.113
2500	-2.312*	0.778	0.639	0.029
5000	-1.888	0.151	0.969	0.469
7500	-1.902	0.215	0.940	0.416
10000	-1.819	0.262	0.906	0.503

Table notes.
Nominal CL-20 concentrations are reported. Analytically determined initial concentrations of CL-20 in SSL soil and percent recovery after 1, 2, 3, 4, 6, and 8 months of incubation are shown in Table 15. *Significantly different (p=0.029).

These results indicate indirectly that soil biotic activity that controls the rate of litter decomposition in SSL soil used in our study was either unaffected or stimulated by exposure to CL-20 in amended soils up to and including 10,300 mg kg^{-1} initial concentration (8238 mg kg^{-1} final after 8 months). This conclusion is also supported by the findings of the microcosm study, which assessed the effects of CL-20 on the microinvertebrate community in the same test units that were used in the litter decomposition assay. This microcosm study showed that overall composition of microarthropod community in SSL soil was not affected by exposure to CL-20 based on the number of taxonomic group present in the individual treatments after 12 weeks. The equally represented oribatid mites and collembola were the second most abundant individual groups among microarthropods (prostigmatid mites were most abundant), and jointly comprised approximately 40% of the microarthropod community throughout the study. Grazing activity of the oribatid mites and collembola provide the greatest contribution to litter decomposition among studied groups of microarthropods. Furthermore, dominance of bacterivore and fungivore nematodes among the nematode community, and the increases in their absolute numbers that were sustained throughout the study, suggest indirectly that availabilities of their respective food sources (bacteria and fungi), were also unaffected-or-increasing in soil CL-20 treatments. This is important because bacteria and fungi play the primary role in litter decomposition though production of extracellular enzymes involved in organic matter breakdown.

The direct evidence supporting the hypothesis that the soil microbial community can tolerate or be stimulated by exposure to CL-20 come from the studies by Gong et al. (2004). Those authors demonstrated in definitive studies with similar SSL soil that indigenous soil microorganism were unaffected by exposure to CL-20 up to and including 10,000 mg kg^{-1} (Gong et al. 2004). Using two microbial assays, including dehydrogenase activity (DHA) and potential nitrification activity (PNA), Gong et al. (2004) showed that DHA was actually stimulated in 100 mg kg^{-1} CL-20 treatment, PNA was stimulated in the 1,000 mg kg^{-1} CL-20 treatment, and both were stimulated in the 10,000 mg kg^{-1} CL-20 treatment. Taken together, results of our study of Orchard grass litter decomposition and those reported by Gong et al. (2004), show that exposure to CL-20 up to and including 10,000 mg kg^{-1} concentration does not adversely affect microbial activity in SSL soil. Integrating litter decomposition assay into the microcosm study design allowed us to assess the effects of CL-20 on both structure (relative abundance of the soil community groups) and function (litter decomposition, critical process for carbon and nutrient cycling) of the soil ecosystem. Applying this integrated microcosm design to ecotoxicological investigations can improve ecological risk assessment (ERA) by incorporating ecological principles into ERA methodologies.

6.2.8 Derivation of Draft Ecological Soil Screening Levels (Eco-SSL) for CL-20

One of the objectives of this project was to generate toxicity benchmark values for terrestrial plants and soil invertebrates that could be used for developing draft Eco-SSLs for CL-20. Ecotoxicological testing was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Draft Guideline (USEPA 2005). General concepts of this Draft Guideline are summarized in this section of report to assist users in reviewing and interpreting its findings. The Eco-SSLs are screening values that can be used to identify

contaminants of potential concern (COPCs) in soils that require further evaluation in a Baseline Ecological Risk Assessment (ERA), and to eliminate those that do not. Eco-SSLs are concentrations of contaminants in soils that are protective of ecological receptors that commonly come into contact with soil or ingest biota that live in or on soil. For the two groups of ecological receptors plants and soil invertebrates, Eco-SSLs are derived separately. As such, these values are expected to provide adequate protection of terrestrial ecosystems.

The draft Eco-SSLs developed for CL-20, if accepted by USEPA, should be used during Step 2 of the Superfund ERA process, the screening-level risk calculation. It is expected that the Eco-SSLs will be used to screen site soil data to identify whether CL-20 may present potential ecological concern, or does not need to be considered in the subsequent Baseline ERA. The draft Eco-SSLs are intentionally conservative in order to provide confidence that contaminants that potentially present an unacceptable risk are not screened out early in the ERA process. This conservative nature of Eco-SSLs is achieved by using a natural soil type that has properties maximizing bioavailability to ecologically relevant test species, by using growth (for terrestrial plants) or reproduction (for soil invertebrates) measurement endpoints for benchmark derivation, and by relying on a low, EC_{20} (20% reduction) level of the effect on a measurement endpoint for Eco-SSL development.

The draft Eco-SSLs may apply only to sites where terrestrial receptors may be exposed directly or indirectly to contaminated soil. They were derived for the two groups of ecological receptors terrestrial plants, and soil invertebrates. The Eco-SSLs for terrestrial plants consider direct contact of CL-20 in soils and potential uptake, and for soil invertebrates they consider ingestion of soil as well as direct contact exposures. Both exposures were considered under conditions of relatively high bioavailability in SSL soil. By deriving conservative soil screening values protective of these receptor groups, it is assumed that the terrestrial ecosystem will be protected from possible adverse effects associated with soil contamination when used in conjunction with Eco-SSLs developed for avian wildlife and mammalian wildlife (if available).

Soil physical and chemical properties affect the exposure of organisms, including terrestrial plants and soil invertebrates, to contaminants in soils (Alexander 1995; Loehr and Webster 1996; Linz and Nakles 1997). Terrestrial plant and soil invertebrate Eco-SSLs are applicable to all sites where key soil parameters fall within a certain range of chemical and physical parameters (USEPA 2005). They apply to upland aerobic soils where: pH 4 ≤ soil pH ≤ pH 8.5, and the organic matter (OM) content is ≤10% OM. The majority of soil toxicity tests that were reported in literature utilized standard artificial soil with high organic matter content (10%), which limited their usefulness for Eco-SSL derivation. In contrast, our toxicity studies designed to specifically fill the knowledge gap regarding ecotoxicity of energetic material (EM) contaminants in soil, used a natural soil that meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. This was necessary to ensure that draft Eco-SSLs for terrestrial plants and soil invertebrates developed in this project are adequately conservative for a broad range of soils within the specified boundary conditions (USEPA 2005).

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentration of a chemical over those based on nominal concentrations

(USEPA 2005). In this project, the exposure concentrations of CL-20 in soil were analytically determined in all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330A (USEPA 1998) for extraction of EMs from soil and for measuring acetonitrile-extractable chemical concentrations. Comparison of results obtained based on acetonitrile extraction of freshly amended soils showed good agreement between nominal and measured concentrations for CL-20. This confirmed that the soil amendment procedure used in toxicity tests developing ecotoxicological benchmarks for draft Eco-SSL derivation was appropriate, and that the USEPA Method 8330A was efficient for quantifying the amount of CL-20 in soil.

Among the important aspects for a draft Eco-SSL development are selections of test methods and test species for toxicity testing to generate ecotoxicological benchmarks. The USEPA preference for using standardized toxicity assays for generating benchmarks, and ecological relevance of test species used to soil ecosystems, was emphasized in the Eco-SSL guidelines (USEPA 2005). A limited number of terrestrial toxicity tests have been developed, or improved by standardization, by different agencies and organizations since the early 1990s. Leading among these standardizing organizations are: the International Standardization Organization, the American Society for Testing and Materials, Environment Canada, Organization for European Co-operation and Development, the United States Environmental Protection Agency, and the European initiative SECOFASE (Development, Improvement, and Standardization of Test Systems for Assessing **S**ublethal **E**ffects of **C**hemicals **o**n **F**auna in the **S**oil **E**cosystem) with its mandate to develop test systems for the early detection and evaluation of sublethal effects of chemicals on organisms in soil ecosystems. The new or improved test methods developed under the auspices of SECOFASE have been summarized in a handbook of soil invertebrate toxicity tests by Løkke and van Gestel (1998).

After an extensive review of existing standardized test methods, and based on the experience accumulated in the participating laboratories, we selected ASTM standard guide for conducting terrestrial plant toxicity tests (ASTM 1998), and USEPA early seedling growth test (USEPA 1982), for assessing CL-20 effects on terrestrial plants. Our previous studies, including those supported by SERDP (CU-1221 and ER-1210) identified the three plant species most sensitive to EMs tested, and within performance parameters in SSL soil required by the validity criteria for the standardized definitive toxicity tests. These species included a dicotyledonous symbiotic species alfalfa, and two monocotyledonous species Japanese millet and ryegrass.

ISO assays were selected for toxicity testing with soil invertebrates. These assays included ISO 11268-2:1998 *Soil Quality – Effects of Pollutants on Earthworms (Eisenia fetida) – Part 2: Determination of Effects on Reproduction* (ISO 1998a); ISO 16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO 2005); and ISO 11267 *Soil quality — Inhibition of Reproduction of Collembola* (*Folsomia candida*) *by Soil Pollutants* (ISO 1998b). Guidelines for these ISO assays were originally developed for use with Artificial Soil (OECD/USEPA Standard Artificial Soil); however research in our laboratory has shown that each of these bioassays can be successfully adapted for use with natural soils (Kuperman et al. 1999; 2003, 2004, 2005, 2006a,b,c,d; Simini et al. 2003), which was necessary for establishing benchmarks acceptable for use in Eco-SSLs development.

Energetic materials can affect populations of terrestrial plants and soil invertebrates in different ways. These include (1) direct acute toxicity, (2) chronic toxicity such as effects on growth and/or reproduction, (3) indirect toxicity by altering soil structure or physical characteristics, fertility, or chemistry (4) indirect toxicity by adversely affecting nutrient and food supplies, or (5) by affecting predators and parasites. In addition, soil organisms may alter their environment changing the overall bioavailability of chemicals within the soil. No single test can address these types of effects, and a battery of tests is required to reasonably do so. Inclusion of species from different taxonomic groups representing a range of sensitivities, which often correlate with physiologically-determined modes of action and can vary among taxa, was an important consideration for selecting the test battery for Eco-SSL development. The selected species were expected to represent the spectrum of diverse ecological functions that are attributed to organisms comprising soil communities: primary producers, and different functional groups of soil invertebrates. Test species selected for our studies are representative surrogates of the total species that normally inhabit a wide range of site soils and geographical areas (i.e., ecologically relevant). Test invertebrate species used in this investigation actively move through soil, thus ensuring contact with contaminants. Both terrestrial plant and soil invertebrate species tested are sensitive to a wide range of contaminants, and reflect different routes of exposure (e.g., ingestion, inhalation, dermal absorption for soil invertebrates, and uptake from soil solution for plants). It was important for Eco-SSL development, that selected test invertebrate species were amenable to life cycle tests to include most vulnerable developmental stages of the test organisms (e.g., adult survival, or cocoon and/or juvenile production). Finally, the selected terrestrial toxicity tests have been standardized with representative test species and generate reproducible, statistically-valid results, which imparts a greater confidence in the data and generates less uncertainty associated with the decisions and recommendations that are based on the test data.

A draft Eco-SSL for an CL-20:invertebrates pairing was calculated as the geometric mean of EC_{20} toxicity values determined from the individual studies; three toxicity data values generated under specified conditions are the minimum required to calculate an Eco-SSL (USEPA 2005). The draft Eco-SSL derivation process was completed for CL-20 for soil invertebrates. CL-20 was not phytotoxic up to 10,000 mg kg^{-1} nominal (7,856 mg kg^{-1} analytically determined), the highest concentration tested in the Limit Test with the three plant species. Consequently, no draft Eco-SSL for terrestrial plants could be developed for CL-20. Separate draft Eco-SSL values were derived for freshly amended and for weathered and aged CL-20 treatments of SSL soil. Reproduction measurement endpoints in tests with soil invertebrates were more sensitive to CL-20 compared with adult survival. This supports and comports with the Eco-SSL requirement for the use of reproduction endpoints for benchmark development (USEPA 2005). Consequently, reproduction measurement endpoints were used for derivation of draft Eco-SSL for soil invertebrates. These reproduction endpoints included cocoon production and juvenile production for earthworms, and juvenile production for potworms and collembola.

Table 17. Derivation of Draft Eco-SSL value for CL-20 in freshly amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus*

crypticus, and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations of CL-20 in soil (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	0.05	0.03-0.07	
Juvenile Production	0.04	0.02-0.06	
Potworm			0.08
Juvenile Production	0.1	0.06-0.13	
Collembola			
Juvenile Production	0.2	0.12-0.34	

Table 18. Derivation of Draft Eco-SSL value for CL-20 weathered and aged in Sassafras sandy loam soil, using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus*, and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations of CL-20 in soil (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	0.036	0.005-0.067	
Juvenile Production	0.038	0-0.094	
Potworm			0.08
Juvenile Production	0.035	0.025-0.045	
Collembola			
Juvenile Production	1.0	0-4	

Draft Eco-SSLs for soil invertebrates were developed for CL-20 freshly amended into SSL soil and for CL-20 weathered and aged in SSL soil. The calculated draft Eco-SSLs for each CL-20 - receptor are presented in Tables 17 and 18, listed here for sake of comparison and increased understanding of different outcomes based on different experimental conditions.

These Eco-SSL values are unofficial, since USEPA must review experimental designs of studies, the data produced, and its applicability, before accepting benchmarks or deriving Eco-SSL values.

The draft Eco-SSL values were derived using the EC_{20} level of the CL-20 effects on soil invertebrate reproduction measurement endpoints. The preferences for reproduction benchmarks and for low effect level were justified in order to ensure that Eco-SSL values would be protective of majority of ecological receptors in soil, and provide confidence that EM concentrations posing an unacceptable risk are not screened out early in the ERA process. Review of the ecotoxicological benchmarks generated in our studies shows that both requirements, including the use of reproduction effects and the use of EC_{20} response level, were well justified. Reproduction measurement endpoints were more sensitive compared with adult survival in all soil invertebrate tests. The EC_{20} level for reproduction benchmarks generally approximated the ANOVA-based no effect (NOEC) levels for CL-20 in most studies.

Project design was evaluated using the Literature Evaluation Criteria accepted by the Eco-SSL Workgroup and summarized in Table 19. This was done to ensure that draft Eco-SSLs developed by our studies comply with all criteria, and would be expected to obtain the highest score in each category. Such review would also expedite the transition of the results of our investigations to Eco-SSL Workgroup, who will also apply rules of selection to determine the most appropriate benchmarks for establishing the respective Eco-SSL values.

Table 19. Summary of literature evaluation process for plant and soil invertebrate Eco-SSLs (modified from USEPA 2005).

Criteria	Rationale
1: Testing was Done Under Conditions of High Bioavailability.	Bioavailability of metals and polar organic compounds is influenced by pH and soil organic matter, cationic exchange capacity, and clay content. The scoring is intended to favor relatively high bioavailability.
2A: (Laboratory) and **2B:** (field): Experimental Designs for Studies are Documented and Appropriate.	Experimental design can significantly influence the quality of a study. Higher quality studies will use an experimental design sufficiently robust to allow analysis of the test variables and discriminate non-treatment effects.
3: Concentration of Test Substance in Soil is Reported.	The concentration of the contaminant tested must be reported unambiguously.
4: Control Responses are Acceptable**.**	Negative controls are critical to distinguish treatment effects from non-treatment effects.
5: Chronic or Life Cycle Test was Used**.**	Chronic toxicity tests assessing long-term adverse sub-lethal impacts on the life-cycle phases of an organism are considered superior to acute toxicity tests.
6: Contaminant Dosing Procedure is Reported and Appropriate for Contaminant and Test.	Contaminant dosing procedure may affect the outcome of a test. Dosing procedure should include: (A) The form of the contaminant; (B) The carrier or vehicle (e.g., solvent, water, etc.); (C) How the carrier was dealt with following dosing (i.e., allowed to volatilize, controls, etc.); (D) procedure for mixing of soil with contaminant (homogeneity)**.**
7: A Dose-Response Relationship is Reported or can be Established from Reported Data.	Two methodologies that can be used to identify this benchmark concentration. The first method generates a no observed effect concentration (NOEC) and a lowest observed effect concentration (LOEC). The second method uses a statistical model to calculate a dose response curve and estimate an effect concentration for some percentage of the population (EC_p), usually between an EC_5 and an EC_{50}.
8: The Statistical Tests used to Calculate the Benchmark and the Level of Significance were Described.	Statistical tests and results reported in the study should be sufficient to determine the significance of the results.
9: The Origin of the Test Organisms is Described.	The results of a toxicity test can be influenced by the condition of the test organisms. Culture conditions should be maintained such that the organisms are healthy and have had no exposure above background to contamination prior to testing (invertebrates) or detailed information is provided about the seed stock (plants).

Information relevant for each criterion of the evaluation processes is summarized below.

1. A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the EM toxicity for the test species used. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents).

2. Toxicity assays were conducted to determine the effects of CL-20 on soil invertebrates. Testing was designed to specifically meet the requirements for Eco-SSL development. All methods used are documented in relevant sections of this report, and in appendices presenting the detailed account of individual studies. All assays included range-finding tests to bracket CL-20 concentration range for each test species, and definitive tests to determine ecotoxicological benchmarks required for development of draft Eco-SSL values.

3. Nominal concentrations were analytically verified in all definitive test treatments. All ecotoxicological parameters were established using measured chemical concentrations for each treatment level.

4. Each toxicity test was appropriately replicated and included negative (no chemicals added), positive (reference chemical), and carrier (acetone) controls. Test validity criteria were used in all definitive assays. Validity criteria in definitive toxicity tests with terrestrial plants specified minimal percent germination in negative controls for each species tested, and the quality control limit for EC_{50} values in positive control (boric acid). Validity criteria for negative controls in the definitive toxicity tests with soil invertebrates specified minimal percent adult survival, minimal number of juveniles produced, boundaries for coefficient of variation for reproduction, and percent reduction in positive control (beryllium sulfate) from negative control, determined for reproduction measurement endpoint based on the baseline established for the laboratory cultures of earthworms, potworms, and collembola.

5. All toxicity tests were based on the assessments of CL-20 effects on reproduction of soil invertebrates in addition to acute endpoint, adult survival.

6. Soil amendment procedures were documented and included the form of CL-20 used, analytical purity, procedures for preparation of treatment concentrations using acetone carrier, time allowed to volatilize acetone in chemical hood, and duration of 3-dimentional mixing to ensure the homogeneity of CL-20 incorporation in test soil.

7. Measurement endpoint data were analyzed using regression models to establish concentration-response relationships for each CL-20-test species:measurement-endpoint pairing. The EC_{20} and EC_{50} values for cocoon/juvenile production in the soil invertebrate assays were determined using SYSTAT 11 software. The EC_{20} parameter is preferred for deriving Eco-SSL values. The EC_{50}, a commonly reported value, was included to enable comparisons of the results produced in this study with results reported by other researchers.

8. Statistical tests included regression analyses and Analysis of Variance (ANOVA). Linear and nonlinear regression analyses were performed using SYSTAT 11 software. Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined. ANOVA was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values (when possible). Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values. Student's *t*-Test (two-tailed) with significance level set at $p \leq 0.05$ was used in the Limit Tests with plants using EXCEL software (Microsoft Corporation 1997).

9. All soil invertebrate test species used in toxicity assays came from cultures maintained by the Environmental Toxicology laboratory, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA.

Review of the information provided for each criterion shows that experimental design of ecotoxicological investigations complied with all screening criteria used by the Eco-SSL Workgroup during literature evaluation processes for selecting soil invertebrate benchmarks for Eco-SSL development. The Draft Eco-SSL values developed in this project will be provided to the Ecological Soil Screening Level (Eco-SSL) Workgroup for review. Results will undergo quality control review by the Eco-SSL Task Group and their assessment of compliance with rules of selection before determining which benchmarks may be included in the Eco-SSL database, and before acceptance as Ecological Soil Screening Levels (Eco-SSLs) for CL-20 for soil invertebrates.

6.3 Effects of CL-20 on Aquatic Organisms

The chronic toxicity of neat CL-20 to aquatic species was investigated using the test species algae, daphnia, and fathead minnows, in independent tests incorporating direct addition of CL-20 to produce target exposure concentrations. Stock suspension of CL-20 was prepared by adding CL-20 directly to respective media. Ultrasonic treatment of CL-20 in the respective aquatic test media created uniform suspensions of CL-20. Samples were withdrawn from the suspensions of CL-20 and diluted with media to appropriate concentrations. Aquatic toxicity tests with daphnia were also conducted using leachates collected from intact soil cores amended with nominal CL-20 concentrations 100 and 10000 mg kg^{-1}. Chemical analyses were done to determine the soluble fraction of CL-20 in media, providing ecotoxicological parameters to be reported in mg L^{-1}.

6.3.1 Effects of CL-20 on Green Alga *Selenastrum capricornutum*

The green algae were exposed to CL-20 added directly into media. A stock suspension of 100 mg L^{-1} CL-20 was serially diluted to analytically determined treatment concentrations of 4.7, 10.5, 21.5, 46.2, and 94.2 mg L^{-1}. Algal growth was affected after 96h and 10d within the CL-20 concentration range tested (Figure 11). The toxicological benchmark values established in the 96h and 10d exposures are shown in Table 20.

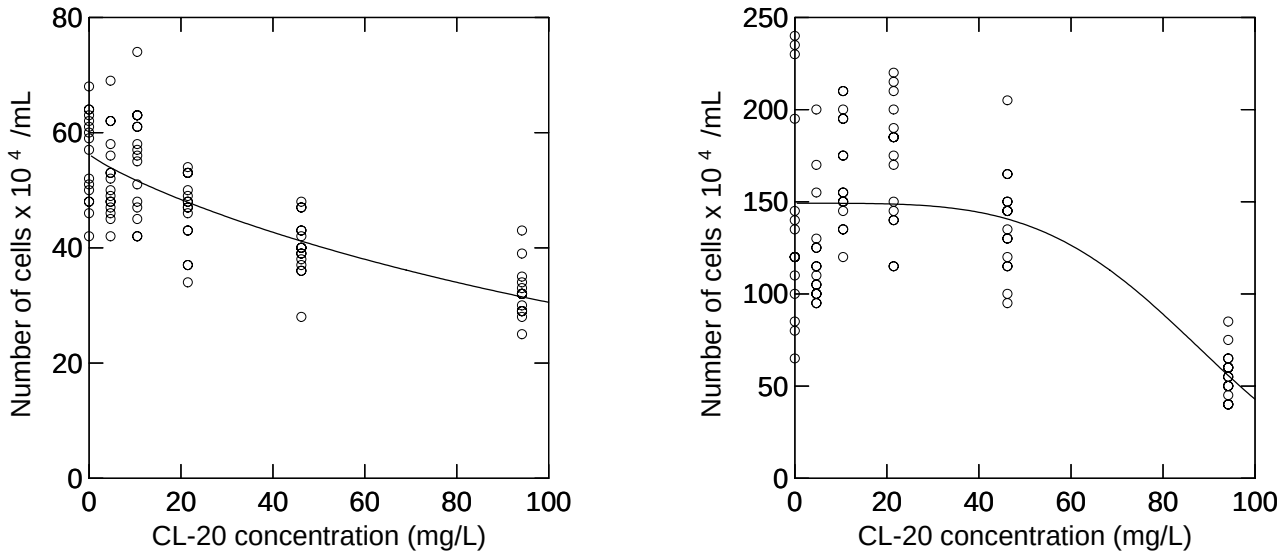

Figure 11. Effects of CL-20 on *Selenastrum capricornutum* in directly amended test media determined using data from the 96 h (left) and 10 d (right) Algal Growth Inhibition Assays.

Notes (Figure 11): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic (Gompertz) model $Y = a \times e([\log(1\text{-}p)]\times[C/\mathrm{ICp}]^{b})$; ($r^2 > 0.933$). Method detection limit = 0.05 mg L^{-1}.

Table 20. Summary of toxicological benchmarks (mg L^{-1}) established for CL-20 in directly amended test media using Algal Growth Inhibition Assays with *Selenastrum capricornutum*.

Exposure time	NOEC	LOEC	IC_{20}	IC_{50}
96 hours	10.5	21.5	31	116
p or 95% CI	0.815	<0.0001	18-45	88-143
10 days	46.2	94.2	65	86
p or 95% CI	0.876	<0.0001	43-87	76-96

Table notes: Concentrations of CL-20 are based on acetonitrile extraction of CL-20 and determination by high performance liquid chromatography (HPLC) using U.S. Environmental Protection Agency Method 8330A (USEPA 1998). Method detection limit = 0.05 mg L^{-1}; IC = inhibition concentration; CI = confidence intervals**;** NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration.

The results of this study showed that CL-20 was moderately toxic to *S. capricornutum* in directly-amended growth media. Extending the exposure time from 96 h to 10 days did not significantly affect (95% CI) algal growth suggesting that toxicity of CL-20 to *S. capricornutum* is not likely to increase with time, in case of accidental release in the environment. The factors affecting CL-20 solubility in aqueous systems and the mechanisms of CL-20 toxicity to *S. capricornutum* both require further investigations because all toxicological benchmarks established in these studies were well above the reported aqueous solubility of CL-20 in pure water (3.7 mg L^{-1} at 25°C).

A Limit 10-d Algal Growth Assays was conducted using aqueous extract from freshly amended SSL soil using a single nominal CL-20 concentration of 1000 mg kg^{-1}. Analytically determined CL-20 concentration in this aqueous extract was 8 mg L^{-1}. Data for the treatment group were compared to data from the control soil extracts prepared from SSL soil without CL-20 amendment. Algal growth was significantly affected (stimulated) in aqueous extract from CL-20 amended SSL ($p<0.05$), producing an unbounded NOAEC of 8 mg L^{-1}.

A Limit 10-d Algal Growth Assay was also conducted with aqueous extract of CL-20 from CL-20 subjected to weathering and aging for 482 d in SSL soil, from a single nominal CL-20 concentration of 1000 mg kg^{-1}. This aqueous extract produced analytically determined CL-20 exposure concentration in the aqueous test media of 4.3 mg L^{-1}. The *S. capricornutum* growth was not significantly affected in aqueous extract from CL-20 amended SSL ($p>0.05$), producing an unbounded NOEC of 4.3 mg L^{-1}.

6.3.2 Effects of CL-20 on the Daphnid *Ceriodaphnia dubia*

Ceriodaphnia Survival and Reproduction assays with directly amended media were conducted using stock suspensions of 32 mg L^{-1} CL-20 (nominal) prepared with ceriodaphnia media, and filtered through 0.45-micron filters. The resulting analytically determined CL-20 concentration in ceriodaphnia media was 3.0 mg L^{-1}. The filtered stock was serially diluted to analytically determined CL-20 concentrations 0.2, 0.4, 0.7, 1.5, and 3.0 mg L^{-1} for use in the Ceriodaphnia Survival and Reproduction assays. After 7 days of exposure, the NOEC and LOEC values for reproduction were 0.4 and 0.7 mg L^{-1}, respectively. Concentration-response relationship for neonate production after the 7-d exposure determined by nonlinear regression analysis (Logistic Gompertz model, Figure 12) produced the IC_{20} and IC_{50} values of 1.2 and 1.9 mg L^{-1}, respectively (Table 21).

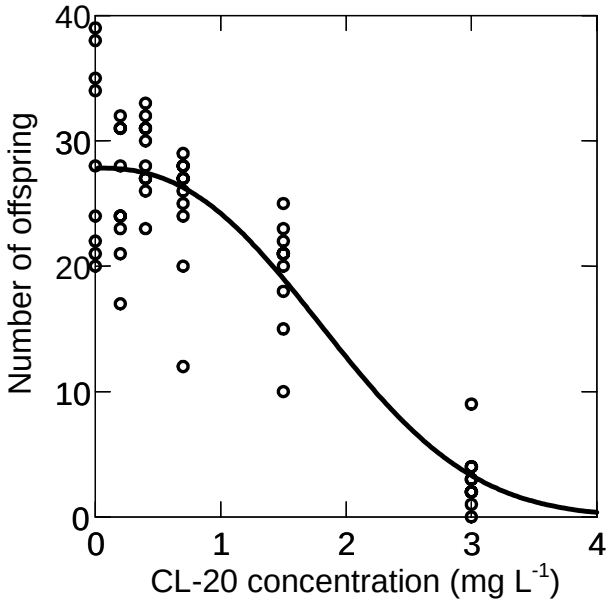

Figure 12. Effect of CL-20 on reproduction of *Ceriodaphnia dubia* in directly amended media.

Notes (Figure 12): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic Gompertz model (r^2=0.960).

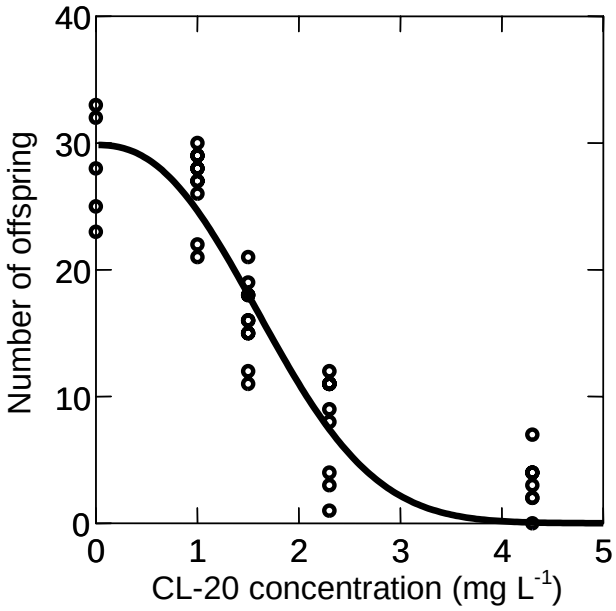

Figure 13. Effect of CL-20 on reproduction of *Ceriodaphnia dubia* in extracts from Sassafras sandy loam soil freshly amended with CL-20.
Notes (Figure 13): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic Gompertz model (r^2=0.966).

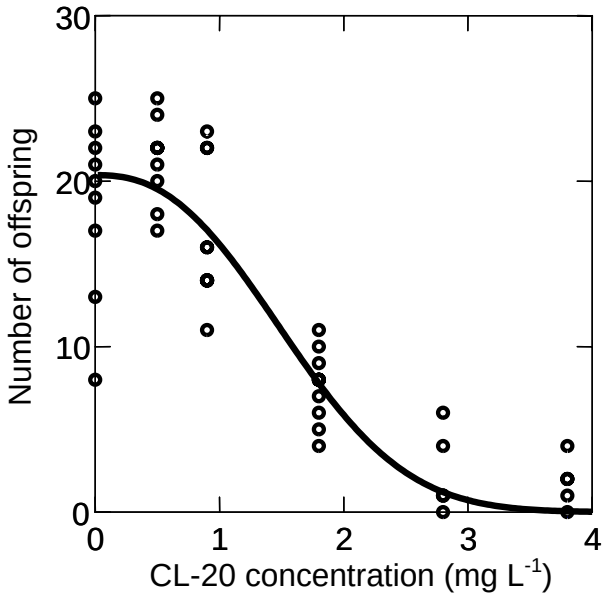

Figure 14. Effect of CL-20 on reproduction of *Ceriodaphnia dubia* in soil extracts of CL-20 subjected to weathering and aging in Sassafras sandy loam.

Notes (Figure 14): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic Gompertz model (r^2=0.947). Weathering and aging of CL-20 included exposing hydrated amended soil in open glass containers in the greenhouse to alternating wetting and drying cycles for 482 days.

Ceriodaphnia Survival and Reproduction assays were also conducted using extracts produced from SSL soil freshly amended with CL-20 to produce nominal concentrations of 5, 7.5, 10, 20, 30 and 40 mg kg^{-1}. The resulting analytically determined CL-20 concentrations in the extracts were 1, 1.5, 2.3, 4.3, 5.7 and 7 mg L^{-1}. After 7 days of exposure, the resulting NOEC and LOEC values for reproduction were 1.0 and 1.5 mg L^{-1}, respectively. Based on neonate production, the IC_{20} and IC_{50} values determined by nonlinear regression analysis (Logistic Gompertz model, Figure 13) were 1.1 and 1.8 mg L^{-1}, respectively (Table 21). There were significant survival effects on *C. dubia* exposed to aqueous extracts having CL-20 concentration from 5.7 to 7.0 mg L^{-1}. Based on survival, the 7-d LC_{50} value was 4.2 (3.5-5.0, 95% CI) mg L^{-1}.

Additional assays were conducted using aqueous soil extract of CL-20 subjected to weathering and aging for 482 d in SSL soil, from the nominal CL-20 concentration of 100 mg kg^{-1}. The extract was serially diluted with ceriodaphnia media to produce analytically determined CL-20 concentrations of 0.5, 0.9, 1.8, 2.8, and 3.6 mg L^{-1}. Based on neonate production, the IC_{20} and IC_{50} values determined by nonlinear regression analysis (Logistic Gompertz model, Figure 14) were 1.0 and 1.6 mg L^{-1}, respectively. The NOEC and LOEC values were 0.9 and 1.8 mg L^{-1}, respectively (Table 21).

Table 21. Summary of toxicological benchmarks for CL-20 established in the 7-d Ceriodaphnia Survival and Reproduction assays with *Ceriodaphnia dubia*.

Exposure Type	IC_{20} mg L^{-1}	IC_{50} mg L^{-1}	NOEC mg L^{-1}	LOEC mg L^{-1}
Direct Amendment	1.2	1.9	0.4	0.7
95% CI or *p*	0.9-1.5	1.6-2.2	0.714	0.025
Aqueous Extracts: FA	1.1	1.8	1.0	1.5
95% CI or *p*	0.9-1.3	1.6-1.9	0.189	<0.0001
Aqueous Extracts: WA	1.0	1.6	0.9	1.8
95% CI or *p*	0.7-1.3	1.4-1.8	0.087	<0.0001

Table notes:
Direct Amendment = CL-20 added directly to algae media. Aqueous Extracts: FA = water extracts from SSL soil freshly amended with CL-20. Aqueous Extracts: WA = water extracts from SSL soil amended with CL-20 subjected to weathering and aging procedure for 482 days. Concentrations are based on determination by HPLC-UV using USEPA Method 8330A. Method detection limit was 0.06 mg kg^{-1}. NOEC = no observed effect concentration; LOEC = lowest observed effect concentration; IC = inhibiting concentration; CI = confidence intervals.

Aquatic toxicity tests with *C. dubia* were conducted using leachates collected from CESMU soil columns amended with nominal CL-20 concentrations 100 and 10,000 mg kg^{-1}. Leachates from replicate soil columns of individual treatments collected during 7 weeks of administering synthetic precipitation to intact soil cores were combined to produce sufficient quantity of leachate required for each day of testing. The leachates did not require centrifugation and were used directly (not serially diluted; leachate changed every 24 h). Test results showed that after the 7-d exposure of ceriodaphnia to leachates, there was 100% mortality in leachates from the nominal 10,000 mg kg^{-1} treatment and 80% mortality in leachates from the nominal 100 mg kg^{-1} treatment (significantly different from control treatment at $p<0.05$). There was no reproduction by *C. dubia* in leachates from either of these CL-20 treatment groups. Similar results were produced using leachates collected during week 14 of administering synthetic precipitation to intact soil cores. Toxicity testing of leachates produced during week 21 revealed 100% ceriodaphnia mortality in leachate produced from the nominal 10,000 mg kg^{-1} treatment and 70% mortality in leachate from the nominal 100 mg kg^{-1} treatment. A total of 6 offspring (significantly different from control treatment at $p<0.05$) were produced in leachate from the nominal 100 mg kg^{-1} treatment. Similar results were produced using leachates collected during weeks 28 and 35. The ceriodaphnia mortality in leachates from the nominal 100 and 10,000 mg kg^{-1} treatments ranged from 70 to 100% with no offspring produced. The ceriodaphnia exposed to control soil leachates (weeks 7, 14, 21, and 28) showed significant decrease ($p<0.05$) in offspring productivity when compared to the growth media controls; however, productivity of ceriodaphnia exposed to control soil leachate produced only from week 35 was not significantly different ($p>0.05$) from the productivity in control media. These effects were seen due to using synthetic rainwater to produce leachate, especially when compared to media designed for *C. dubia*. These effects were not seen when using ceriodaphnia media to extract CL-20 from amended soils. See Table 22 for a summary of the CESMU leachate aquatic toxicity study. The measured concentration of CL-20 in the leachate showed an overall increase over time.

Table 22. Survival and reproduction of *Ceriodaphnia dubia* in leachates from intact Sassafras sandy loam soil cores amended with CL-20 and subjected to synthetic precipitation for 35 weeks.

CL-20 treatment/ Collection time	Determined Leachate CL-20 mg L^{-1}	Number of Offspring*	Mortality %
Media control; 7 weeks	BDL	26(2.1)	0
0 mg kg^{-1}; 7 weeks	BDL	7.3(1.7)	10
100 mg kg^{-1}; 7 weeks	0.02	0	80
10,000 mg kg^{-1}; 7 weeks	1.0	0	100
Media control; 14 weeks	BDL	23.7(0.5)	0
0 mg kg^{-1}; 14 weeks	BDL	2.1(0.8)	0
100 mg kg^{-1}; 14 weeks	0.25	0	0
10,000 mg kg^{-1}; 14 weeks	1.23	0	80
Media control; 21 weeks	BDL	19.3(2.3)	0
0 mg kg^{-1}; 21 weeks	BDL	11.1(1.4)	0
100 mg kg^{-1}; 21 weeks	0.35	0.6(0.3)	70
10,000 mg kg^{-1}; 21 weeks	1.30	0	100
Media control; 28 weeks	BDL	22.4(0.8)	0
0 mg kg^{-1}; 28 weeks	BDL	1.2(0.3)	10
100 mg kg^{-1}; 28 weeks	0.28	0	93
10,000 mg kg^{-1}; 28 weeks	1.70	0	100
Media control; 35 weeks	BDL	22.7(2.0)	0
0 mg kg^{-1}; 35 weeks	BDL	20.1(1.2)	0
100 mg kg^{-1}; 35 weeks	0.3	0	100
10,000 mg kg^{-1}; 35 weeks	1.5	0	70

Table notes:
Data are means and standard errors (SE); n=10. Leachates were collected from replicate CESMU intact SSL soil cores during the 35-week study. Concentrations of CL-20 are based on determination by HPLC-UV using USEPA Method 8330A. BDL = below detection limit. Method detection limit was 0.06 mg kg^{-1}.

6.3.3 Effects of CL-20 on Fish *Pimephales promelas*

Fathead Minnow Survival and Growth assays with directly amended media were conducted using stock suspensions of 50 mg L^{-1} CL-20 (nominal) prepared with fish media, and filtered through 0.45-micron filters. The resulting analytically determined CL-20 concentration

was 3.8 mg L^{-1}. The filtered stock was serially diluted to analytically determined CL-20 concentrations 0.9, 1.8, 2.8, 3.8 mg L^{-1} for use in the assays. After 7 days of exposure, the NOEC and LOEC values for growth were 1.8 and 2.8 mg L^{-1}, respectively. Concentration-response relationship for growth after the 7-d exposure determined by nonlinear regression analysis (Logistic Gompertz model, Figure 15) produced the IC_{20} and IC_{50} values 2.0 and 2.7 mg L^{-1}, respectively. The 7-d LC_{50} value for survival was 2.0 mg L^{-1} (Table 23).

Fathead Minnow Survival and Growth assays were also conducted using aqueous extracts produced from SSL soil freshly amended with nominal CL-20 concentrations 5, 7.5, 10, 20, 30, 40, 50, and 100 mg kg^{-1}. The resulting analytically determined CL-20 concentrations in the extracts were 0.6, 0.9, 1.3, 2.6, 3.6, 4.5, 5.7 and 7 mg L^{-1}. After 7 days of exposure, the resulting NOEC and LOEC values for growth were 1.3 and 2.6 mg L^{-1}, respectively. The resulting growth IC_{20} and IC_{50} values determined by nonlinear regression analysis (Logistic Gompertz model, Figure 16) were 1.4 and 2.9 mg L^{-1}, respectively. There were significant survival effects on fathead minnows exposed to aqueous extracts having CL-20 concentration from 3.6 to 7 mg L^{-1}. Based on survival, the 7-d LC_{50} value was 3.4 mg L^{-1} (Table 23).

Additional assays were conducted using aqueous soil extract of CL-20 subjected to weathering and aging for 482 d in SSL soil, from the nominal CL-20 concentration of 100 mg kg^{-1}. The extract was serially diluted to produce analytically determined CL-20 concentrations of 0.5, 0.9, 1.8, 2.8, and 3.6 mg L^{-1}. The resulting growth EC_{20} and EC_{50} values determined by nonlinear regression analysis (Logistic Gompertz model, Figure 17) were 2.2 and 3.0 mg L^{-1}, respectively. The NOEC and LOEC values for growth were 0.9 and 1.8 mg L^{-1}, respectively (Table 23).

Table 23. Summary of toxicological benchmarks for CL-20 established in the 7-d Fathead Minnow Survival and Growth assays with *Pimephales promelas.*

Exposure Type	IC_{20} mg L^{-1}	IC_{50} mg L^{-1}	LC_{50} mg L^{-1}	NOEC mg L^{-1}	LOEC mg L^{-1}
Direct Amendment	2.0	2.7	2.0	1.8	2.8
95% CI or *p*	0.9-3.2	2.0-3.3	1.7-2.3	0.411	0.019
Aqueous Extracts: FA	1.4	2.9	3.4	1.3	2.6
95% CI or *p*	0.4-2.4	2.1-3.7	3.1-3.7	0.102	0.011
Aqueous Extracts: WA	2.2	3.0	ND	0.9	1.8
95% CI or *p*	1.7-2.6	2.8-3.3	ND	0.587	0.045

Table notes:
Direct Amendment = CL-20 added directly to algae media. Aqueous Extracts: FA = water extracts from SSL soil freshly amended with CL-20. Aqueous Extracts: WA = water extracts from SSL soil amended with CL-20 and subjected to weathering and aging procedure for 482 days. ND = Concentrations are based on determination by HPLC-UV using USEPA Method 8330A. Method detection limit was 0.06 mg kg^{-1}. NOEC = no observed effect concentration; LOEC = lowest observed effect concentration; IC = inhibiting concentration; LC = lethal

concentration; CI = confidence intervals. ND = parameter could not be determined within CL-20 concentration range tested.

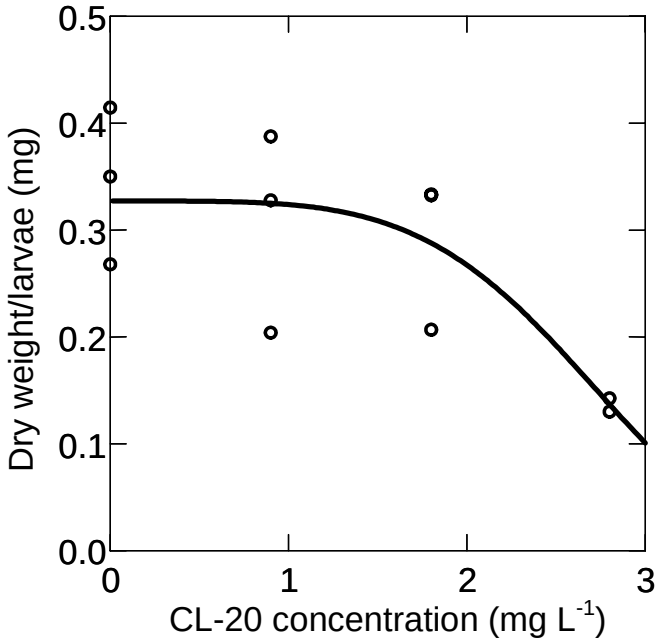

Figure 15. Effect of CL-20 on *Pimephales promelas* growth in directly amended media.

Notes (Figure 15): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic Gompertz model (r^2=0.958).

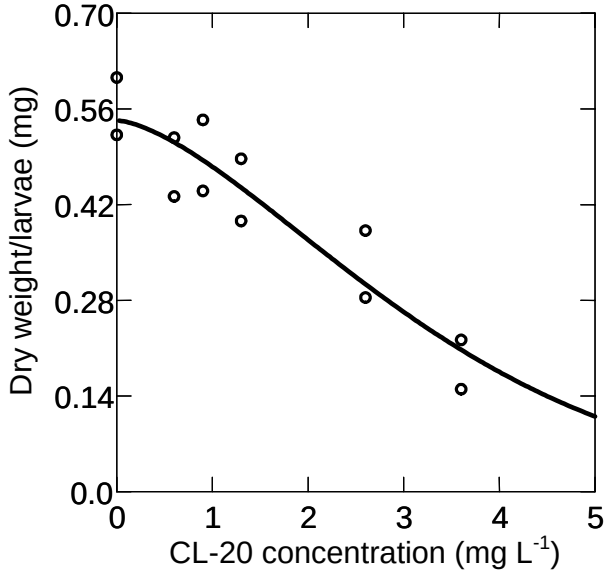

Figure 16. Effect of CL-20 on *Pimephales promelas* growth in extracts from Sassafras sandy loam soil freshly amended with CL-20.
Notes (Figure 16): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic Gompertz model (r^2=0.987).

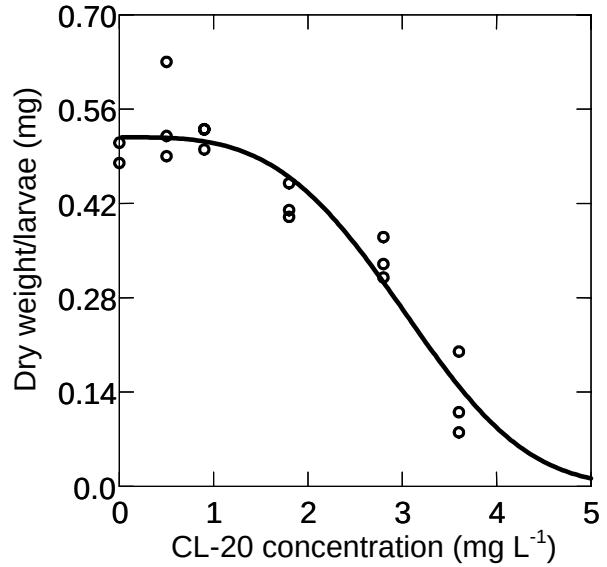

Figure 17. Effect of CL-20 on *Pimephales promelas* growth in soil extracts of CL-20 subjected to weathering and aging in Sassafras sandy loam soil.
Notes (Figure 17): Concentration-response relationships were established using analytically determined CL-20 concentrations and logistic Gompertz model (r^2=0.989). Weathering and aging of CL-20 included exposing hydrated amended soil in open glass containers in the greenhouse to alternating wetting and drying cycles for 482 days.

6.3.4 Discussion

Aquatic bioassays included direct amendments of CL-20 to test media and aquatic elutriates from SSL soil amended with various concentrations of CL-20 to partially simulate the potential toxicity of CL-20 to aquatic receptors resulting from direct CL-20 release into the aquatic environment, and from contaminated surface soil runoff, respectively. The exposures of test organisms to aqueous extracts of soil containing CL-20 better represents conditions if surface runoff from fields happens to contaminate bodies of water or contaminated soil erodes into waterways, while results from direct amendment better represent instances if direct contamination of bodies of water occurs.

Results from definitive toxicity assays with aquatic species showed that green alga *S. capricornutum* was the least sensitive organism to CL-20 toxicity among the three aquatic species tested. Algal growth was actually stimulated by exposure to CL-20 up to 8 mg L^{-1}, the highest concentration tested in the 10-d assay with elutriates from CL-20 amended SSL soil, is in agreement with studies conducted by Gong et al. (2004). Gong reported no adverse effect of CL-20 on *S. capricornutum* up to 3.6 mg L^{-1} in the 96-h assay using only soluble CL-20 exposures. Inhibition of algal growth occurred in our studies only after exposing *S. capricornutum* to CL-20 concentrations ranging from 21.5 to 94.2 mg L^{-1} for 10 days. In addition, studies conducted by Burton and Turley (1994) exposing *S. capricornutum* for 96 h to RDX concentrations up to 36.9 mg L^{-1} have shown the NOEC and LOEC for cell growth to be 0.5 and 4.8 mg L^{-1} respectively, indicating that CL-20 is less toxic to algae than RDX.

Although low toxicity effects of CL-20 on algal growth can in part be interpreted as an indication of relatively low risk of environmental impact from an accidental release of this compound, comprehensive assessment should also consider broader ecological scale by including investigation of the indirect effects of such release. One such possible indirect effect is the increased risk of algal blooms from stimulated growth of algae in response to CL-20 contamination of water bodies and corresponding depletion of dissolved oxygen, both of which are detrimental to aquatic ecosystems. Examples abound of highly damaging effects resulting from increased nutrient supply, or contamination that leads to eutrophycation of aquatic habitats.

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Studies by Peters et al. (1991) reported the NOEC and LOEC for RDX toxicity to ceriodaphnia to be 3.6 and 6.0 mg L^{-1}, respectively; and after 7 days of exposure to 16.4 mg L^{-1} RDX, there were no effects on ceriodaphnia survival. Comparing RDX to our results on the

toxicity of CL-20 in directly amended aqueous media (Table 21), CL-20 appears to be an order of magnitude more toxic to ceriodaphnia than is RDX.

In contrast to the effects on autotrophic *S. capricornutum*, CL-20 was highly toxic to heterotrophic aquatic species *C. dubia* and *P. Promelas*, both when exposed in directly amended test media or in aquatic elutriates from CL-20 amended SSL soil. The toxicity benchmarks for reproduction or growth ranged from 1 to 3 mg L^{-1} for both species, and were quite similar for exposures in directly amended media and in aquatic elutriates from CL-20 amended SSL soil (Tables 21 and 23). Comparison of our results to other studies was not possible at the time of preparation of this report because CL-20 is a new energetic material and no ecotoxicological data were available for these species in published literature. Our results show that both IC_{20} and IC_{50} toxicity benchmarks for *C. dubia* reproduction and *P. promelas* growth were within the range of CL-20 solubility in water (3.16 mg L^{-1} at 20ºC, Monteil-Rivera et al. 2004). This indicates that relevant ecological receptors can be at risk from exposure to CL-20 from different routes of potential CL-20 input, including direct release into aquatic habitats and from contaminated surface soil runoff.

The transport and fate studies (Section 4.4) also indicate that CL-20 can be persistent in the vadose zone of soil, even in soil that has both low clay and organic matter contents. Furthermore, sorption of aqueous CL-20 is relatively small (K_d = 0.02 to 3.83 cm^3 g^{-1}; 2 cm^3 g^{-1} for SSL), which results in only slight retardation relative to water movement and enables dissolved CL-20 to move relatively easily and quickly through unsaturated and saturated sediments (Szecsody et al. 2004). After 35 weeks of CL-20 migrating through the soil column, the aquatic toxicity effects remained significant (p=0.05) indicating that the aquatic community may also be affected by percolation of water through a soil system, providing an additional rough of CL-20 input.

The relatively high toxicity to heterotrophic aquatic species, and potentially stimulating effect on autotrophic algae, suggest that a substantial release of CL-20 into either terrestrial or aquatic ecosystems may cause significant ecological damage in affected sites. This information should be considered by the manufacturer, potential users, risk assessors, and future site managers, during proposed periods of transition to CL-20 for military products that currently use energetic cyclic nitramines RDX and HMX.

6.4 Transport and Fate of CL-20 in Intact Sassafras Sandy Loam Cores

Initial CL-20 concentrations in the nominal 100 mg kg^{-1} and 10,000 treatments were determined to be 98.4 and 9,555 mg kg^{-1}, respectively. Infiltration and percolation of simulated rainfall caused CL-20 to migrate downward in soil resulting in a decrease in CL-20 concentration in the amendment concentrate (0-2 cm; CL-20 in SSL soil) during the 35-week study. Concentration of CL-20 increased over time in the underlying soil layers, although the greatest percentages and corresponding concentrations of CL-20 were retained primarily in the upper 12 or 5 cm of soil in the nominal 100 or 10,000 treatments, respectively (Figures 18 and 19). More than 80% of the initial (98.4 and 9,555 mg kg^{-1}, respectively) CL-20 remained in the

upper 12 cm in the nominal 100 mg kg^{-1} treatment, and in the upper 5 cm in the nominal 10,000 mg kg^{-1} treatment (Figures 20 and 21). CL-20 concentrations dramatically declined at depths greater than 12 or 5 cm of soil in the nominal 100 or 10,000 mg kg^{-1} treatments, respectively; with the 12 or 5 cm depths, respectively, having less than 1% of initial CL-20 amended atop column surface (Figures 20 and 21). In terms of percentage of initial CL-20 amended atop column surface, both the nominal 100 and 10,000 mg kg^{-1} treatments generally had concentrations corresponding to less than 0.2% below the respective depths of 12 and 5 cm; with CL-20 concentrations below the depths of 12 and 5 cm, respectively, generally decreasing further with respect to depth in both treatments (Figures 20 and 21).

Similar patterns of migration in response to simulated rainfall occurred with respect to CL-20 concentrations by depth in both treatments, but at substantially different magnitudes. These migration patterns are in part related to the rates of dissolution of CL-20, and its subsequent passage in solution downward through the soil columns. The decrease in CL-20 concentration over time in the uppermost surface soil, from the initial concentrations, is evident in both the 100 and 10,000 mg kg-1 nominal treatments (Figures 18 and 19).

In the nominal 100 mg kg^{-1} treatment, the CL-20 concentrations at the 2 cm soil depth regularly decrease at each subsequent sampling time during the entire 35-week period. The 3 cm depth shows the evolution of the process of dissolved CL-20 partitioning between soil and pore water as dissolved CL-20 migrates downward, and the resulting retardancy of CL-20 migration due to this partitioning. After 7 weeks of simulated rainfall the CL-20 concentration at 3 cm depth is greater than 20 mg kg^{-1}, and the CL-20 concentration in soil at this depth peaks after 14 weeks. Incremental loading of the soil at each greater depth continues to occur over time. This pattern of incremental loading is most clearly shown at the 7 cm depth, but occurs at each subsequent depth to a lesser degree.

In the nominal 10,000 mg kg^{-1} treatment, decrease in CL-20 concentration in the uppermost surface soil, the initial source of CL-20, occurs within the initial 7 weeks of simulated rainfall. However the initial loading level (9,555 mg kg^{-1}) is so great that the rate of CL-20 dissolution limits the levels of subsequent diminishment of CL-20 at near-surface depths, with approximately stable concentrations at the 2 cm and 3 cm depths, respectively, from 14 to 35 weeks of simulated rainfall. The nominal 10,000 mg kg^{-1} treatment then exhibits a pattern of CL-20 incremental loading at greater depths, similar to that in the nominal 100 mg kg^{-1} treatment. Corresponding to the nominal 100 mg kg^{-1} treatment, this pattern of incremental loading is most clearly shown at the 7 cm depth, and again occurs at subsequent depths to lesser degrees. In the regions of incremental loading of CL-20, the nominal 10,000 mg kg^{-1} treatment resulted in approximately ten-fold greater concentrations compared to the nominal 100 mg kg^{-1} treatment at similar depths, resulting from the rate of dissolution of CL-20, and the resulting retardancy of CL-20 migration due to partitioning of dissolved CL-20 between soil and pore water.

The transport of CL-20 in soil was primarily due to solubilization and subsequent partitioning between soil and pore water, and this can be investigated further by interpretation of the resulting distribution of CL-20 and its relative accessibility (Figures 22 and 23), which also affects CL-20 bioavailability in soil. Water-extractable CL-20 after 35 weeks accounted for approximately 50 and 80 percent of acetonitrile extractable CL-20 at soil depths exceeding 20

cm in nominal 100 or 10,000 mg kg^{-1} treatments, respectively. Lower percentages of water-extractable CL-20, compared to acetonitrile extractable CL-20, at lesser subsurface depths also indicates that the soil has some carrying capacity for CL-20, perhaps due to sorption. However, the continuing migration of CL-20 even in the nominal 100 mg kg^{-1} treatment indicates that this carrying capacity cannot be very great, and ultimately does not substantially inhibit long-term migration.

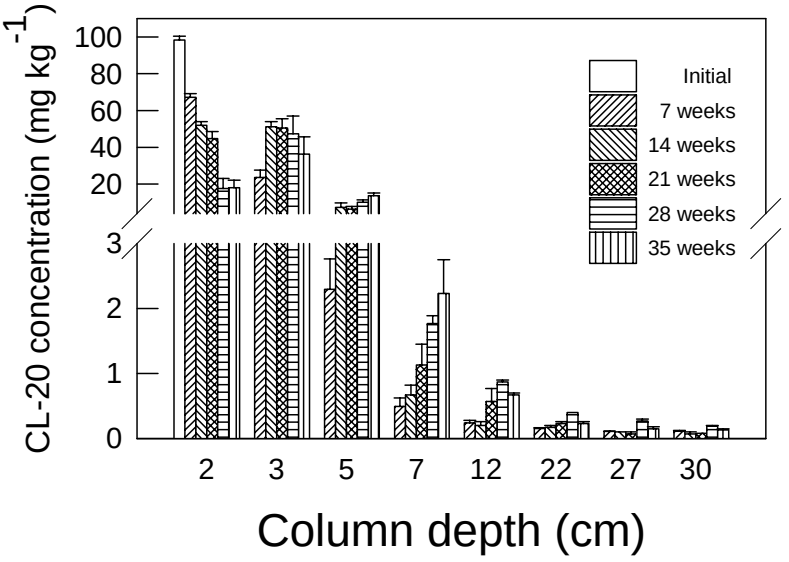

Figure 18. Concentrations of CL-20 in soil sections of the intact soil cores determined in the 98.4 mg kg^{-1} treatment during the 35-week study. Data are means and standard errors (n=9).

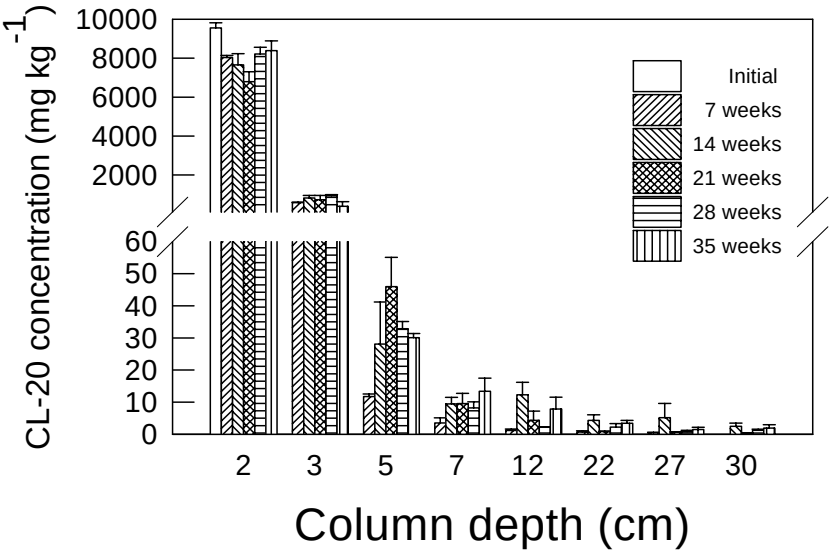

Figure 19. Concentrations of CL-20 in soil sections of the intact soil cores determined in the 9,555 mg kg^{-1} treatment during the 35-week study. Data are means and standard errors (n=9).

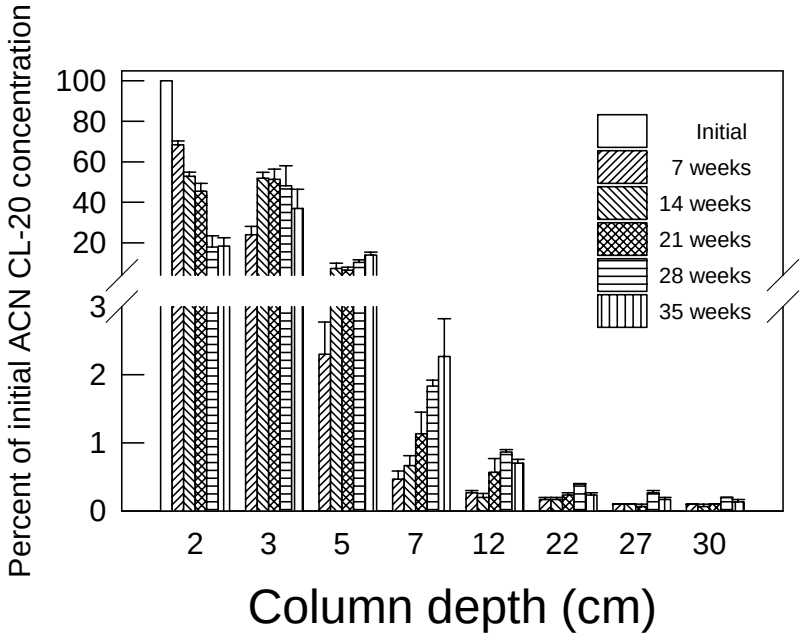

Figure 20. Recovery of CL-20 in soil sections of the intact soil cores expressed as percent of the initial 98.4 mg kg^{-1} concentration amended atop column surface. Data are means and standard errors (n=9).

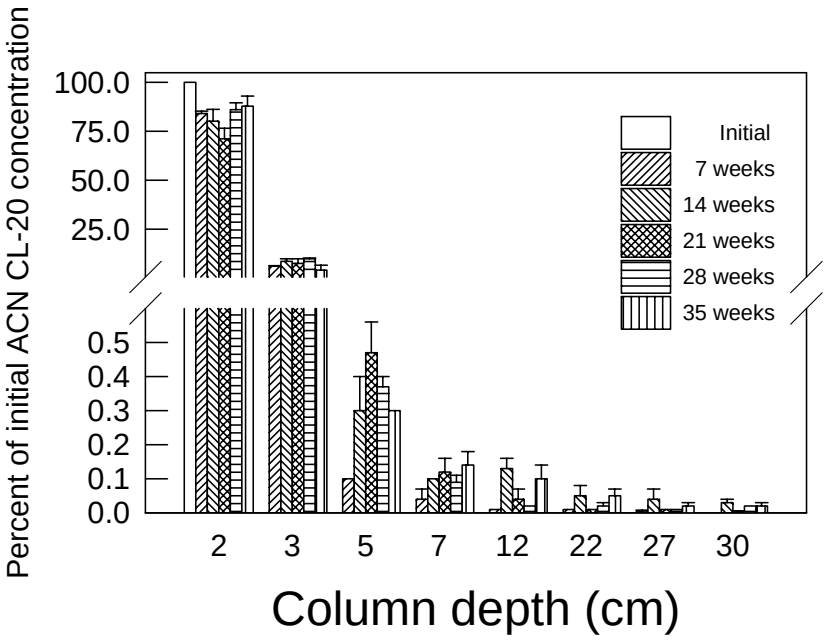

Figure 21. Recovery of CL-20 in soil sections of the intact soil cores expressed as percent of the initial 9,555 mg kg^{-1} concentration amended atop column surface. Data are means and standard errors (n=9).

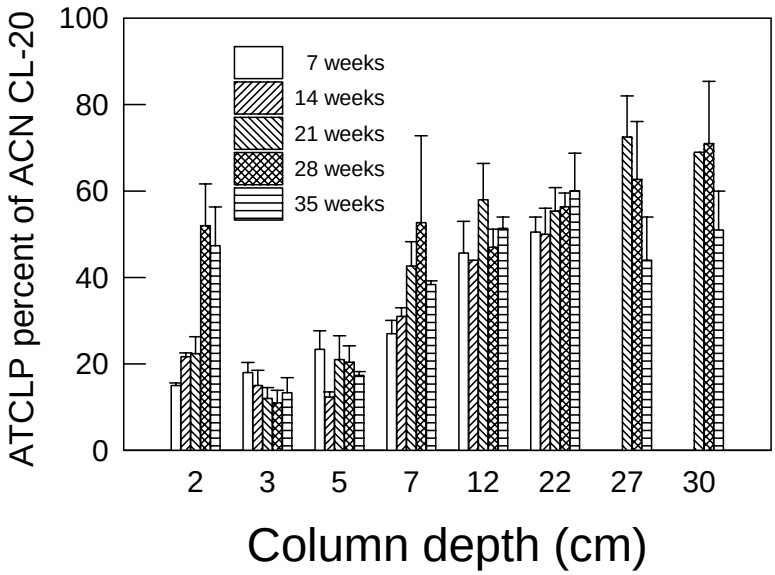

Figure 22. Transport of solubilized CL-20 in soil sections of the intact soil cores, amended atop column surface with 98.4 mg kg^{-1} CL-20, and expressed as percent of acetonitrile (ACN) extractable fraction. ATCLP = Adopted Toxicity Characteristic Leaching Procedure. Data are means and standard errors (n=9).

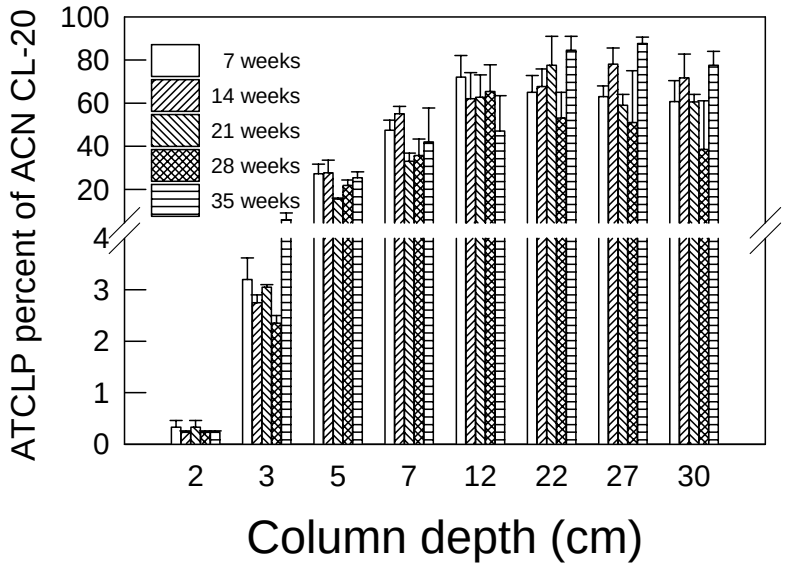

Figure 23. Transport of solubilized CL-20 in soil sections of the intact soil cores, amended atop column surface with 95,555 mg kg^{-1} CL-20, and expressed as percent of acetonitrile (ACN) extractable fraction. ATCLP = Adopted Toxicity Characteristic Leaching Procedure. Data are means and standard errors (n=9).

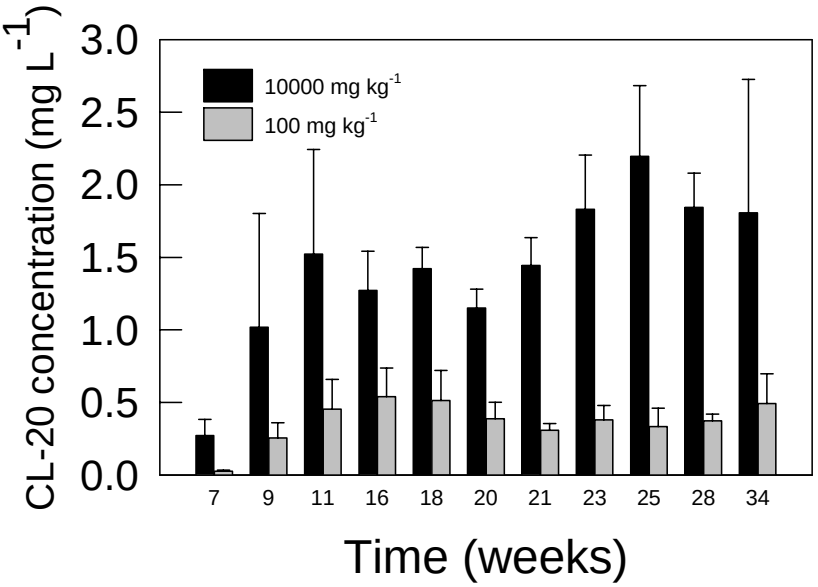

Figure 24. Concentrations of CL-20 in leachates from the intact soils cores amended atop column surface with 98.4 or 95,555 mg kg^{-1} CL-20.
Notes (Figure 24): Data are means and standard errors (n=2-12). The number of columns available for producing leachates changed during the chronosequential harvesting of soil columns, producing variable n throughout the study period.

Concentrations of CL-20 in the aqueous leachates remained below the reported limit of CL-20 solubility, but occurred in measurable concentrations from the initial sampling at 7 weeks, onward (Figure 24). Concentrations of CL-20 in the aqueous leachates were dependent on initial CL-20 concentration in the amendment, and increased over time becoming relatively stable after 11 weeks. This indicates CL-20 has a reasonably high potential for slow but continuing migration in soil, and ultimately the transport over time of solubilized CL-20 to groundwater. This also indicates that factors enhancing the rate of dissolution of CL-20, or its aqueous solubility, will also enhance its potential for transport into groundwater.

The fate of CL-20 that partitions into water was also assessed by analyzing the plant tissues of *Lolium perenne* L. (perennial ryegrass) harvested from the intact SSL soil core columns at the end of the 35-week study. CL-20 concentrations in plant shoot tissues were used to determine the bioaccumulation potential of CL-20 in *L. perenne*. Results of the plant bioaccumulation tests are shown in Table 24. Mean CL-20 concentrations in shoots of perennial ryegrass were 9 and 209 mg kg^{-1} in soil treatments initially amended with 98.4 or 9,555 mg kg^{-1} CL-20, respectively. The bioaccumulations factors (BAFs), calculated by dividing mean CL-20 concentration in plant tissue by mean CL-20 concentration in soil, were 0.09 and 0.02 for 98.4 and 9,555 mg kg^{-1} treatments, respectively (Table 24). Bioaccumulation potential for CL-20 in ryegrass, is therefore, predicted to be low.

Table 24. Concentrations of CL-20 in soil and shoots of perennial ryegrass used in establishing the bioaccumulations factors (BAFs).

Nominal CL-20 in surface soil (mg kg^{-1})	Determined CL-20 in surface soil (mg kg^{-1})	Determined CL-20 in plant (mg kg^{-1})	S.E.	BAF
0	BDL	BDL	-	-
100	98.4	9	2.67	0.09
10000	9555	209	60.54	0.02

Table Notes: SE=Standard Error of mean (n=3). BDL= below detection limit (0.012 μg mL^{-1}).

These investigations showed that potential transport and fate of CL-20 in the soil vadose zone was effectively assessed using the CESMU method with SSL soil that has "high" relative bioavailability for organic EMs. Precipitation (simulated rainfall) caused CL-20 to migrate downward in the soil, with concentrations of CL-20 rapidly decreasing with increasing depth of the soil core. Exceptionally small quantities of CL-20 migrated below the 12-cm soil depth of the intact soil core, indicating that CL-20 is not expected to migrate to substantial depth in solid crystal form. Transport of CL-20 in the soil vadose zone was primarily due to solubilization and subsequent partitioning between soil and pore water. Concentrations of CL-20 in soil leachates never reached the CL-20 limit of solubility but increased over 11 weeks, becoming relatively stable at approximately 0.5 and 2 mg L^{-1} in nominal 100 and 10,000 mg kg^{-1} soil treatments, respectively. These results suggest a reasonably high potential for long-term

transport of CL-20 to groundwater. Soil physico-chemical parameters that enhance the carrying or sorptive capacity of soil for CL-20 will retard (but not eliminate) CL-20 migration. However, soil factors that enhance the rate of dissolution of CL-20 in soil, or its aqueous solubility in soil pore water, will increase its potential for transport into groundwater. The BAF values of 0.09 and 0.02 established in studies with ryegrass grown in 98.4 or 9,555 mg kg^{-1} CL-20 treatments for 35 weeks, respectively suggest low bioaccumulations potential for CL-20 in plants.

7. Conclusions

This project was undertaken to assess the potential impacts of the release of CL-20 into the environment. The investigation produced experimental data yielding benchmarks for environmental fate, transport through the soil vadose zone, and ecotoxicological effects of CL-20 on terrestrial plants, soil organisms, and aquatic species. Ecotoxicological effects of CL-20 were determined using both standardized single-species toxicity assays for soil invertebrates, plants and aquatic species, and an experimental approach designed to assess community-level effects on soil invertebrates and on litter decomposition, which integrates the exposure impacts on carbon and nutrient cycling in the soil ecosystem. The effects of weathering and aging of soil:chemical mixtures was incorporated into the experimental design to better assess the bioavailability potential of CL-20 undergoing biotic and abiotic degradation and transformation processes. Toxicity endpoints were correlated with analytically determined chemical concentrations to develop ecotoxicological benchmark data for CL-20 based on concentration-response relationships that describe its bioavailability to ecologically relevant soil and aquatic biota. Transport and fate of CL-20 in soil were determined using a modified standardized intact soil-core microcosm system (CESMU) designed to assess the risks of CL-20 release in the soil vadose zone, including its potential for downward migration in solid crystal or solubilized forms, the potential for transport to groundwater, and the potential for bioaccumulation into terrestrial plants. Ecotoxicological testing was specifically designed to meet the criteria required for Eco-SSL derivation. A natural soil, Sassafras sandy loam, was used in all toxicity tests. Sassafras sandy loam (SSL) had low organic matter and clay contents, fulfilling the USEPA Eco-SSL requirement for using soil with characteristics that support relatively high contaminant bioavailability for plant or soil invertebrate testing.

Results of our phytotoxicity studies showed that CL-20 did not adversely affect growth or seedling emergence of *Medicago sativa* L. (alfalfa), *Echinochloa crusgalli* L. (Japanese millet), and *Lolium perenne* L. (perennial ryegrass) in SSL soil up to and including 7,856 mg kg^{-1}. These results were obtained for terrestrial plant species that represent a wide variety of plant genera, and habitats. Alfalfa is a dicotyledonous species. Perennial ryegrass and Japanese millet are monocotyledonous species. Alfalfa and perennial ryegrass are crop plants, whereas Japanese millet grows naturally and can tolerate relatively wet habitats. These factors in combination with our test results suggest that CL-20 is relatively non-toxic to the majority of terrestrial plants.

Standardized single-species toxicity tests with soil invertebrates showed that toxicity of CL-20 to the earthworm *Eisenia fetida*, enchytraeid worm *Enchytraeus crypticus*, or collembolan *Folsomia candida* in natural soil (SSL) was orders of magnitude greater compared

with that of the currently used explosives RDX, HMX, and TNT. Our findings of increased toxicity to *E. crypticus* of soil containing CL-20 weathered and aged in soil clearly show that additional studies are required to investigate the toxicity of the CL-20 degradation products. Analogously, further investigation of the potentially more-toxic degradation products of CL-20 in soil should also have a weathering and aging component, so that the persistence and long-term impact of the ecotoxicity of these degradation products may also be assessed. Resolving CL-20 degradation pathways that lead to formation of toxic products, their fate in aerobic soils, and assessment of the individual toxicities of degradation products to soil receptors require further investigations to better understand the mechanisms of CL-20 toxicity in the soil vadose zone.

We applied the toxicity benchmark values for CL-20 effects on soil invertebrates established in these studies to derive draft Eco-SSL values, for use in Ecological Risk Assessment in the event of accidental release of CL-20 in the environment. Draft Eco-SSL values were derived using the conservative EC_{20} level for the CL-20 effects on soil invertebrate reproduction measurement endpoints determined from standardized toxicity tests. Our experimental data indicate that plant or soil invertebrate Eco-SSL value of 0.08 mg kg^{-1} CL-20 can provide adequate protection for the majority of ecological receptors in soil.

The results of microcosm studies showed that indigenous microarthropod and nematode communities exhibit distinctly different sensitivities to CL-20 in soil. Total nematodes were either unaffected or increased in CL-20 treatments. In contrast, CL-20 adversely affected the microarthropod community, evidenced by significantly decreased total numbers in CL-20 treatments after 4 and 8 weeks of exposure. The overall taxonomic and trophic structure of the indigenous soil microarthropod or nematode communities was not affected by exposure to CL-20 after 12 weeks; however further analysis of community structure revealed greater sensitivities to CL-20 of predatory mesostigmatid mites and predatory nematodes. These decreases in respective predatory groups of the microinvertebrate community can potentially disrupt the soil food web structure, as a result of exposure to CL-20.

Chronosequential harvesting of subset replicate microcosm units allowed us to assess potential alterations in CL-20 bioavailability and toxicity to soil microinvertebrate community due to weathering and aging of CL-20 in situ. The 12-week study revealed that toxicity of CL-20 decreased overtime for Collembola, prostigmatid mites, and predatory nematodes, but briefly increased for oribatid mites after 8 weeks before returning to its initial level after 12 weeks. These results showed the complexity of possible interactions among the physicochemical fate processes during weathering and aging of CL-20 in soil, changes in bioavailability of parent material and its possible degradation products, and the soil receptor-specific sensitivities of diverse groups of the soil invertebrate community.

Toxicity data for soil microarthropods and predatory nematodes were comparable with mortality data established in the standardized single-species toxicity tests with soil invertebrates. Overall, this study showed that community-level assessment and analysis of trophic structure of soil microfauna using a microcosm assay were sufficiently sensitive measures of chemical toxicity, and suggest that microcosm assay can bridge the gap between single-species toxicity tests and field studies. A microcosm assay can also provide the means for

validating the ecotoxicity data from standardized laboratory tests and improve ERA by incorporating ecological principles into ERA methodologies.

Results of litter decomposition study indicated indirectly that soil biotic activity that controls the rate of litter decomposition in SSL soil used in our study was either unaffected or stimulated by exposure to CL-20 up to and including 10,300 mg kg^{-1} initial (8238 mg kg^{-1} final after 8 months) concentration. This conclusion is supported by the findings of other researchers (Gong et al. 2004) and by the results of the microcosm study, which showed that overall composition of microarthropod community and total abundance of nematodes in SSL soil were not affected by exposure to CL-20 for 12 weeks.

Ecotoxicological data established in this project indicate that the release of CL-20 to aquatic ecosystems can pose a significant ecological threat. Results of definitive aquatic toxicity assays showed that relevant ecological receptors can be adversely impacted by exposure to CL-20 concentrations at and above 1 mg L^{-1}. Risk to aquatic species is significant from potential direct release into aquatic habitats, and from contaminated surface soil runoff or erosion of contaminated soil into water bodies. Transport of solubilized CL-20 through the soil vadose zone from contaminated surface soil will pose an additional risk to resident aquatic receptors at CL-20 concentrations in groundwater as low as 0.02 mg L^{-1}. Overall results of this investigation strongly indicate that accidental release of CL-20 into the environment can be detrimental to populations of aquatic species. This information should be considered by the manufacturer, potential users, risk assessors, and future site managers, during proposed periods of transition to CL-20 for military products that currently use RDX, HMX, or TNT.

Our transport and fate study showed that precipitation (simulated rainfall) caused CL-20 to migrate downward in soil, with concentrations rapidly decreasing with increasing depth in soil. Exceptionally small quantities of CL-20 migrated below the 12-cm soil depth of the intact soil core, indicating that CL-20 is not expected to migrate to substantial depth in solid crystal form. Transport of CL-20 in soil was primarily due to solubilization and subsequent partitioning between soil and pore water. Concentrations of CL-20 in soil leachates never reached the CL-20 limit of solubility but increased over 11 weeks, becoming relatively stable at approximately 0.5 and 2 mg L^{-1} in 98.4 and 9,555 mg kg^{-1} soil treatments, respectively. These results suggest a reasonably high potential for slow continuous transport of CL-20 to groundwater. The bioaccumulation factors of 0.09 and 0.02 established in studies with ryegrass grown in 98.4 or 9,555 mg kg^{-1} CL-20 treatments for 35 weeks, respectively suggest low bioaccumulations potential for CL-20 in terrestrial plants.

A portion of the results of this project have been published (or accepted for publication) in peer reviewed journals, and additional manuscripts are in preparation. Two Technical Reports on individual studies of this project have been prepared, and additional Technical Reports are in the final stages of preparation. Principal findings of this project have been presented at national and international professional meeting, including Joint Army-Navy-NASA-Air Force (JANNAF) meetings and were published as Report 2004-0187CY in proceedings of JANNAF meeting in Seattle, Washington, 26-29 July, 2004. In addition, ecotoxicological data for CL-20 will be included in the book *Ecotoxicology of Explosives* co-

edited by G.I. Sunahara, J. Hawari, G. Lotufo, and R.G. Kuperman. This book is currently scheduled for publication by *CRC Press* in 2006.

Transition of our findings is also being facilitated through the Technical Cooperation Program (TTCP). TTCP is an international organization that collaborates in defense scientific and technical information exchange; program harmonization and alignment; and shared research activities for the five nations Australia, Canada, New Zealand, the United Kingdom, and USA. Dr. Kuperman is Leader of the Key Technical Area (TTCP-KTA 4-32) entitled *Development of Environmental Threshold Values for Defense Sites Contaminated with Energetic Materials*. Ecotoxicological data established in this and other SERDP-funded projects were presented at the Annual Meetings of TTCP Weapons Technical Panel TP-4 in Australia, the United Kingdom, and the USA. Data transition was further aided by organizing the International Workshop *Ecotoxicology of Energetic Materials* that was held in Washington DC, 28 November, 2005, with assistance of SERDP/ESTCP. Participants from Canada, Sweden, and the U.S. representing Government, academia, and industry were briefed on the key findings of this project, and identified the current gaps of knowledge in the field of ecotoxicology of energetic materials, and prioritized areas requiring further research.

The toxicity benchmark values for CL-20 and reports detailing these studies will be provided to the Ecological Soil Screening Level Workgroup for review. Results will undergo quality control review by the Eco-SSL Task Group before inclusion in the Eco-SSL database, and before acceptance for derivation of Ecological Soil Screening Level (Eco-SSL) for CL-20. Dr. Checkai, Co-PI of this project, is a Co-Chair of the Eco-SSL Task Group and a member of the Eco-SSL Workgroup Steering Committee. Dr. Kuperman, PI of this project, is a member of the Eco-SSL Task Group. Both Dr. Checkai and Dr. Kuperman will provide a direct conduit for the transitioning data generated during this research to USEPA for the development of Eco-SSL for CL-20.

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APPENDIX A

List of Technical Publications.

Articles published in peer-reviewed journals.

Kuperman, R.G., Mónica J. B. Amorim, M.J.B., Römbke, J. Lanno, R., Checkai, R.T., Dodard, S.G., Sunahara, G.I., Scheffczyk, A. (accepted). Adaptation of the Enchytraeid Toxicity Test for use with natural soil types. *European Journal of Soil Biology*.

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Technical Reports

Anthony, J.S., Davis, E.A., Haley, M.V., Kolakowski, J.E., Kurnas, C.W., Phillips, C.T., Simini, M., Kuperman, R.G., and Checkai, R.T. (2004). HPLC Determination of hexanitrohexaazaisowurtzitane (CL-20) in Soil and Aqueous Matrices. Technical Report ECBC-TR-403. U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. November 2004.

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Conference Proceedings

Kuperman, R.G., Checkai, R.T., Simini, M., Haley, M.V., Phillips, C.T., Anthony, J.S., Kolakowski, J.E., Davis, E.A., Chester, N.A., and Kurnas, C.W. (2004). Toxicity benchmarks for the new energetic material CL-20 for terrestrial and aquatic ecological receptors. Report 2004-0187CY. In: *Proceedings of Joint Army-Navy-NASA-Air Force Meeting*. Seattle, Washington, 26-29 July, 2004.

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Kuperman, R.G., Checkai, R.T., Phillips, C.T., Simini, M., Anthony, J.S., Kolakowski, J.E., and Kurnas, C.W. 2005. Effects of CL-20 on the indigenous soil microarthropod community and litter decomposition. The 2005 SERDP Partners in Environmental Technology Technical

Symposium & Workshop, Washington, DC, November 29 - December 1, 2005 (published abstract).

Kolakowski, J.E., Checkai, R.T., Simini, M., Kuperman, R.G., Phillips, C.T., and Kurnas, C.W. 2005. Analytical precision and quality control: determining concentrations of explosives in environmental media. The 2005 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, November 29 - December 1, 2005 (published abstract).

Phillips, C.T., Checkai, R.T., Kuperman, R.G., Simini, M. 2005. Toxicity of CL-20 to the indigenous community of soil nematodes. The 2005 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, November 29 - December 1, 2005 (published abstract).

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